DIPLOMARBEIT

Titel der Diplomarbeit

Evaluation of novel, nanotechnological biosensor-chips for monitoring of bacterial spoilage: Evaluation of sensor sensitivity and correlation with currently used microbiological testing methods and their improvement

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Verfasser: Mirza Ibrišimović

Matrikel-Nummer: 0305404

Studienrichtung (lt. Studienblatt): Molekulare Biologie

Betreuer: Univ. Prof. Dr. Fritz Pittner

Wien, am 05.05.2009

Aknowledgement

I would like to thank at the first place to my dear parents, Senahid and Safija, and my dear sister Nadira, for their love, financial support and for being my most important backing all this time. Without you all my achievements would not be possible.

Thank you dear Fritz and Gisi for the oppourtinity to work on this topic, for your continuous encouragement and support. It was pleasure knowing you and I shall be always grateful for everyting that you have done for me.

I would also like to thank to my dear lab collegues: Ulrich, Margit, Haifa, Martina and Helmut, for the comfortable working atmosphere.

Content

Abstract

The aim of this work was to contribute to the development of a simple and cheap biomimetic sensor providing reasonable sensitivity and selectivity to indicate the bacterial infection in real time monitoring combined with a memory effect that cannot easily be corrupted. In contrast to the sensor whose design relates to the phenomenon of “anomalous absorption”, which can best be described as thin film enhanced absorption [1], the sensor presented in this work is simpler in its setup. The biosensor developed here consists of two different layers: (i) mirror layer (Ni-Cr composition called Inconnel) and, (ii) biomimetic polymer [poly (lactic-coglycolic acid), PLGA], which can be degraded by microbial lytic enzymes. Bacterial induction for excretion of lipolytic enzymes was done under addition of different substrates (tryptone, sunflower oil, glucose, lactose, etc.) as carbon and energy source.

Testing foods for the presence of microorganisms is a very time consuming and often expensive process therefore intelligent packaging materials with integrated real-time sensor, which respond to microbial contamination of food products, would be an excellent possibility to highlight pollution of foods prior to use.

Concerning meat spoilage, the total bacteria count and also the amount of secreted lipolytic enzymes are of high importance. Gram-negative bacteria as *Pseudomonas* often secrete proteolytic and lipolytic enzymes which are major causes of food decay. Since the environmental conditions have an important influence on the secretion of lipolytic enzymes, we examined the growth behavior of *Pseudomonas, Enterobacter, Salmonella and Proteus* strains which were isolated from spoiled pork meat. It could be shown that spoilt meat contains up to 10^6-10^8 cfu/mm^3 and that the highest amount of secreted enzymes (aminopeptidase) can be found when bacteria are in log phase in concentration of 10^7 cfu/mm^3.

Zusammenfassung

Ziel dieser Diplomarbeit war, Beiträge zur Entwicklung eines einfachen und billigen biomimetischen Sensors mit entsprechender Sensitivität und Selektivität zum „real-time“ Monitoring von mikrobiellem Verderb von Nahrungsmitteln oder Kosmetika zu leisten. Im Gegensatz zu den Sensoren die auf der Basis anomaler Absorption entwickelt wurden [1], ist der hier präsentierte Sensor viel einfacher in seinem Design.
Der neu entwickelte Biosensor besteht aus zwei verschiedenen Schichten: (i) Spiegelschicht (eine Ni-Cr Legierung, bekannt als Inconnel) und (ii) einer biomimetischen Polymerschicht aus [poly (lactic-coglycolic acid), PLGA], die durch das Einwirken lipolytischer Enzyme abgebaut wird. Unter Zugabe von verschiedenen Substraten (Trypton, Sonnenblumenöl, Glucose, Lactose, usw.) als Kohlenstoff- und Energiequelle, war es möglich die Sezernierung von lipolytischen Enzymen in Bakterien zu provozieren.

Die Detektion von für den Lebensmittelverderb verantwortlichen Mikroorganismen ist oft teuer und langwierig. Deshalb könnte ein integrierter „real-time“ Sensor, der bei bakterieller Kontamination ein Signal zeigt, die einfachste Lösung sein, um der Verderb bereits in der Verpackung anzuzeigen.

Beim Fleischverderb sind die Gesamtkeimzahl und die Menge der sezernierten lipolytischen Enzyme von großer Bedeutung. Hauptursache für den Lebensmittelverderb sind Gram-negative Bakterien wie *Pseudomonas,* die oft proteolytische und lipolytische Enzyme produzieren.

Da die Wachstumsbedingungen einen großen Einfluss auf die Sekretion der lipolytischen Enzyme haben, wurde das Wachstum und das Verhalten von *Pseudomonas-, Enterobacter-, Salmonella- und Proteus*-Stämmen, die aus verdorbenem Schweinefleisch isoliert wurden, untersucht. Es hat sich gezeigt dass verdorbenes Fleisch eine Keimzahl zwischen 10^6-10^8 KbE/mm^3 hat. Die größte Menge der Enzyme (überwiegend Aminopeptidase) wird zum Zeitpunkt der log Phase sezerniert, was einer bakteriellen Konzentration von 10^7 KbE/mm^3 entspricht.

Introduction

1. Biosensors

In a biosensor, the sensing element which responds to the substance being measured is biological in nature. It has to be connected to a transducer of some sort so that a visually observable response occurs. Biosensors are generally concerned with sensing and measuring particular chemicals which need not be biological components themselves, although sometimes they are. Many types of microsensors have been produced in last few decades [27-30]. The importance of the biological component is that its interaction with the substrate is highly specific to that substrate alone, thus avoiding interferences from other substances which plague many analytical methods. It may catalyse a reaction involving the substrate (enzyme) or it may bind selectively to the substrate. Biological elements provide the major selective element in biosensors. They must be substances able to attach themselves to one particular substrate but not to others. Four main groups of materials can do this: (i) enzymes, (ii) antibodies, (iii) nucleic acids, and (iv) receptors. The most common component is the enzyme, although other components containing enzymes are often very suitable. These include microorganisms such as yeasts and bacteria.

Antibodies have a different mode of action. They will bind specifically with the corresponding antigen, to remove it from the sphere of activity, but they have no catalytic effect. Despite this, they are capable of developing ultra-high sensitivity in biosensors. Considerable ingenuity is often needed to involve them with the substrate and to provide a signal for the transducer to measure.

Nucleic acids have been much less used so far. They operate selectively because of their base-pairing characteristics. They have great potential utility in identifying genetic disorders, particularly in children. Inside the lipid bilayer plasma membrane surrounding a cell are proteins that traverse the full breadth of the membrane and also have molecular recognition properties. They are known as receptors. They are difficult to isolate, but will bind solutes with a degree of affinity and specificity matching antibodies [2].

1.2 Performance Factors

a.) Selectivity

The most important characteristic of biosensors is their ability to discriminate between different substrates. This is a function of the biological component, principally, although sometimes the operation of the transducer contributes to selectivity.

b.) Sensitivity range

This usually needs to be sub-millimolar, but in special cases can go down to the femtomolar (10^{-15} M) range.

c.) Accuracy

This is usually around ±5 %.

d.) Nature of solution

Conditions such as pH, temperature and ionic strength must be considered.

e.) Time factors

The response time is usually much longer (up to 30 s) than with chemical sensors. The recovery time is the time before a biosensor is ready to analyse the next sample.

It must not be too long-not more than a few minutes. The working lifetime is usually determined by the instability of the biological material. It can vary from a few days to a few months. The Exactech glucose biosensor is, for example, usable for more than one year.

1.3 Role in Industrial Processes

Biosensors can be used in various aspects of fermentation processes in three ways:

(i) Off-line in laboratory;

(ii) off-line, but close to the operation;

(iii) On-line, in real-time

At present, the main real-time monitoring is confined to pH, temperature, CO_2 and O_2. Biosensors which monitor a much wider range of direct reactants and products are available, such as various sugars, yeasts, malt and alcohols and perhaps also undesirable byproducts. Such monitoring could result in improved product quality, increased product yields, checks on tolerance of varations in quality of raw materials, optimized energy efficiency, i.e. improved plant automation, and less reliance on human judgment. There are a wide range of applications in the food and drinks industry generally [2].

1.4 Optical biosensors

Optical biosensors are defined by IUPAC as devices capable of transforming “changes of optical phenomena, which are the results of an interaction of the analyte with the receptor part” [3]. Optical sensors have unique attributes as they can be employed to measure chemicals that are either electro-active or have electro-active reactants or products – but feature some specific optical property, possibly after attachment to a tagged chromophore or fluorophore [4].
Optical biosensors are based on excitation-response measurements of chemicals in a sample, where electromagnetic waves leaking out of the guide interact with chemicals. Specificity to biomolecules is usually brought about by using labelled tags or immobilizing immunoglobulins in the sampling volume. The chemo-optical

transduction layer can either be passive where a physical change of the layer occurs or active where a chemical reaction occurs in the receptor layer.

A large amount of work has gone into optical biosensors. Advantages of these techniques involve the speed and reproducibility of the measurement [5]. Optical biosensors take advantage of the excellent selectivity of the bioreceptor and the outstanding sensitivity of optical detection methods.

Further benefits are the general rigidity, the possibility of multiple use and sometimes even of performance in “real-time”. In many cases the optical sensors do not consume the analyte and are therefore non-destructive analytical methods.

On the contrary there are some disadvantages, like the generally limited long-term stability and restricted dynamic ranges compared to electrodes. Commercial accessories of the optical system are generally very expensive. The primary goal when developing low cost sensors is to employ complexes that can be excited with visible light. Thus, molecules or ions that are actively sensed must have functional optical groups preferably in the visible region so that inexpensive excitation sources and detectors can be used. Biological species to be detected can be proteins, sugars, herbicides, bioluminescent bacteria or DNA.

In spite of the higher costs, optical sensors are attractive because a large number of biochemicals which are not electro-active can be detected by optical excitation response measurements and well-established procedures based on optically active labels can be implemented.

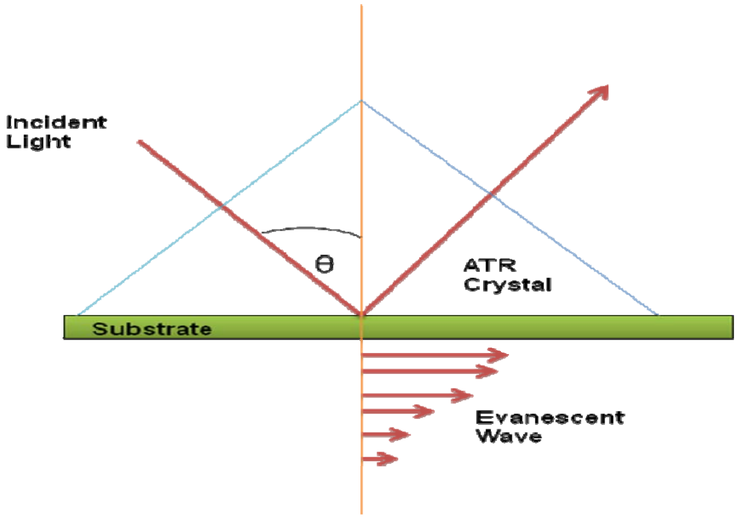


Figure 1. Evanescent wave

All optical biosensors share a common phenomenology in the total internal reflection of light at an interfacial region as their sensing basis. he electric and magnetic field components of light reflected in such a manner do not disappear discontinuously at the reflective boundary but penetrate somewhat into the second medium, decaying with distance from the interface. The existence of light beyond the sharp interfacial boundary is termed *evanescent* in character (Fig.1).
If the interfacial region is constructed to afford a solid–liquid interface constituting an adsorptive surface, the adsorption phenomenon taking place in the region of the evanescent field can alter the measurable characteristics of the total internally reflected light and thus provide the transduction link between biological recognition event and production of a quantifiable signal [6].

2. Psychrophilic Bacteria

Psychrophilic bacteria are distinguished from other bacteria by their ability to grow at low temperatures.Their importance to the dairy industry has been accentuated by the advancements in refrigeration facilities, increased regard for keeping quality, and longer holding times resulting from every-other-day pickup, less frequent home deliveries, and transportation over greater distances. The psychrophiles most frequently encountered are in the geneus *Pseudomonas,* although many bacterial genera contain psychrophilic species.

They are found in water, soil and dirty equipment, and their elimination from milk, meat and dairy products is largely concerned with removing these sources of contamination. Hence, today psychrophiles are defined in essentially four major different ways based on (a) optimum growth temperature, (b) ability to grow at low temperature, (c) the method of enumeration, and (d) criteria which are independent of the temperature. Many bacteria have been observed to develop maximum crops at temperatures below 20°C.

The time necessary for a culture to reach a maximum population at low temperature is impractically long and the variation of maximum populations obtained at various temperatures is impractically small. Heat treated psychrophiles differ from nonheated controls in being more fastidious in their nutrient requirements for initiating growth, in having a more pronounced lag phase at low temperature, and in being more sensitive to the pH of the growth medium [7]. *Salmonella* and *Campylobacter* constitute the most common agents involved in human outbreaks of foodborne disease. In 2005, Salmonella accounted for 64% (3406 out of 5355 outbreaks) of the reported foodborne outbreaks followed by Campylobacter with 9% (494 out of 5355 outbreaks) [8].

Psychrotropic bacteria are important in spoilage of food because as already mentioned they can grow at refrigerator temperatures.

Most psychrotropic bacteria are non-pathogenic, but when growing in dairy products they can cause a variety of off-flavors, including fruity, stale, bitter, putrid, and rancid as well as physical defects. Many psychrotropic bacteria produce heat-resistant proteases and lipases capable of surviving commercial heat treatment such as milk pasteurisation [9].

2.1 Microbial Food Deterioration

Microbial enzymes are major causes of quality deterioration and food spoilage. Understanding of the enzymatic processes which have taken place or may still occur in food is required for making valid, well-profounded shelf-life evaluation. The range of enzyme activity can be much higher than the range of bacterial growth. A major problem is determining the relationship between microbial composition and the appearance of deterioration in food caused by physical, chemical or enzymatic reactions. Even less is known about the activity of microbial enzymes under the effect of the intrinsic factors in food.

Various bacteria have been reported to be exceptionally active producers of protease and lipases. Important proteolytes and lipolytes are aerobic spore-former and

Pseudomonas spp. as a representative genus of the psychrothropic flora. Although spoilage causing flora in food is well characterised there are often differences between the predicted and the actual shelf-life.

One reason could be that the proteolytic and lipolytic activities of the bacteria are not taken into account. It is well known that lipases are able to hydrolase fat, even at very low temperatures [10].

2.2 The Genus Pseudomonas

Pseudomonas comprises a relatively large and important group of gram negative bacteria. Members of this genus occur as free-living organisms in soils, fresh water, marine environments and many other natural habitats. Members also occur associated with plants and animals as normal flora or as agents of disease. Morphological characteristics of this genus are that they are gram negative, non-spore forming, straight or slightly curved rods.

They have very simple nutritional requirements. In the laboratory they grow well in media with some organic matter in solution, at neutral pH, and at temperatures in the mesophilic range. *Pseudomonas* species dissimilate sugars through the Entner-Doudoroff pathway.

Some *Pseudomonas* species can utilize more than 150 different organic compounds as a sole source of carbon and energy and are remarkable for their catabolic diversity. Most importantly, Pseudomonas plasmids confer resistance to many antibiotics and antibacterial agents. Such natural resistance results from its inherent gram-negative cell wall structure, and its propensity to construct protective biofilms in medical settings.

Pseudomonas are important in the balance of nature and also in the economy of human affairs. They are active in aerobic decomposition and biodegradation and play a key role in the carbon cycle. *Pseudomonas* species are renowned for their abilities to degrade compounds which are highly refractory to other organisms, including aliphatic and aromatic hydrocarbons, fatty acids, insecticides and other environmental pollutants.

Pseudomonas and certain other *Pseudomonads* include species pathogenic for humans, domestic animals, and cultivated plants. They cause economically significant crop disease and crop loss world-wide.

3. Industrial Fermentation

A growing microorganism breaks down high molecular weight carbon and energy sources, brings the smaller derivatives into the cell, degrades them to smaller molecules, converts these to amino acids, nucleotides, vitamins, carbohydrates, fatty acids and builds these basic materials into proteins, coenzymes, nucleic acids, mucopeptides, polysaccharides and lipids. Hundreds of enzymes must be made and must act in an integrated manner to avoid total chaos. To do this, regulatory mechanisms have evolved that enable a species to compete efficiently with other forms of life and to survive in nature.

Thus an ideal cell does not overproduce metabolites, regardless of its environment. Some of the important control mechanisms are induction, feedback regulation, catabolite regulation, and energy charge regulation. The fermentation microbiologist, on the other hand, desires a wasteful strain, which will overproduce and excrete a particular compound that can then be isolated.The microbiologist is searching for the microorganism with the weakest regulatory mechanisms. Once the desired strain is found, a development program is begun to improve yields by modification of culture conditions and by mutation. However, the development of fermentations for secondary metabolites, those with no function in growth, still relies mainly on the empirical approach [11].

3.1 Microbial Enzyme Induction

Enzymes are becoming more and more attractive in manufacturing, especially in the food industry and in medicine. The properties of these proteins that lend themselves to extensive use include their rapidity and efficiency of action at low concentrations and under mild conditions of temperature and pH, their lack of toxicity, and the easy termination of their action by mild treatments.

The main reason for the attractiveness of microorganisms as potential enzyme sources is the ease with which enzyme levels may be increased by environmental (such as in our case) and genetic manipulations. Thousandfold increase has been recorded for catabolic enzymes, and biosynthetic enzymes have been increased by several hundredfold.

Other reasons for using microbial cells as sources of enzymes are:

- Enzyme fermentations are economical on a large scale because of short fermentation cycles and inexpensive media.
- Screening procedures are simple and thousands of cultures can be examined in a reasonably short time.
- Different species produce somewhat different enzymes catalysing the same reaction, allowing flexibility with respect to desired operating conditions in the reactor.

Once a good strain is obtained, fermentation parameters are optimized to maximize growth and enzyme production. Very important here are temperature, pH value and nutrition components (sources of carbon, nitrogen, phosphorus, sulphur and mineral salt). Often, especially with extracellular enzymes, there is a need for addition of the surfactants.

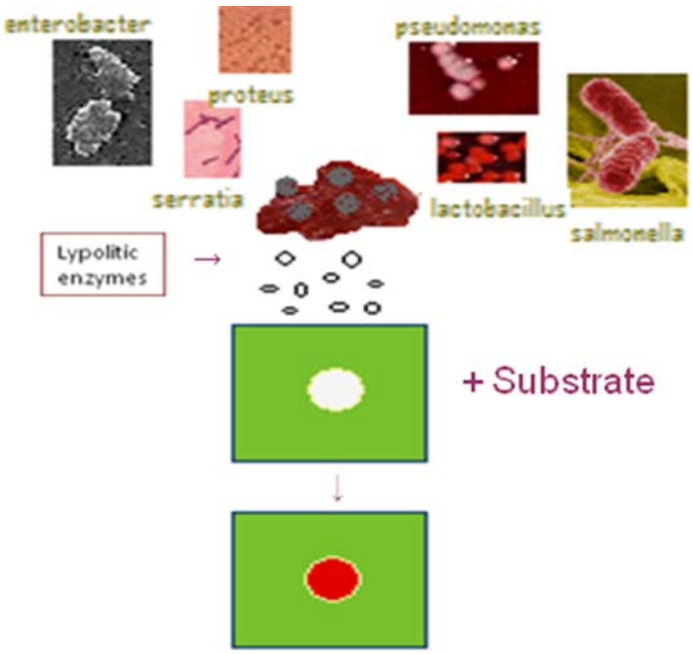


Figure 2. Microbial enzyme production

Non-ionic surfactants are generally preferred over anionic and cationic agents, which are usually toxic to the microoganism. If the fermentation is conducted as a bath culture, the stage of the growth rapidly disappear after reaching their peak activity. Many catabolic enzymes, including those of commercial importance, fall into the inducible category. Typical inducers are substrates such as starch for amylase, sucrose for invertase, lactose for ß-galactosidase or tributyrate, glucose and tryptone for aminopeptidase [11].

Materials and Methods

1. Microbiological Testing Methods

1.2 Agars and Tests for the Isolation, Identification and Total Count of the Food (Meat) Bacteria

1.2.1 Plate Count Agar (*Oxoid, Fluka*)

Plate Count Agar is a microbiological nonselective growth medium for the monitoring of total bacterial growth of the sample.

Composition (g/Liter):

Tryptone 5.0; yeast extract 2.5; dextrose 1.0; Agar 9.0; pH=7.0 ± 0.2.

Preparation:

Suspend 17.5 g of the Plate Count Agar in 1 liter demineralized water and autoclave for 15 minutes at 121°C. In this way prepared plates can be stored for three weeks at 4°C. Total bacterial count is possible to be seen first after 48-72 hours of incubation at 30°C.

1.2.2 ÖNÖZ Agar (*Merck*)

ÖNÖZ Agar is a selective medium for the isolation and identification of *Salmonella* and some other *Enterobacteriacaea.*

Composisition (g/Liter):

Meat extract 6.0**;** yeast extract 3.0; Peptone from meat 6.8; lactose 11.5; sucrose 13.0; bile salt mixture 3.825; tri-sodium citrate 5,5-hydrate 9.3; sodium thiosulfate 5-hydrate 4.25; Sodium green 0.00166; Neutral red 0.022; Aniline blue 0.25; Metachrome yellow 0.47, di-sodium hydrogen phosphate 2-hydrate 1.0; Lphenylalanine 5.0; iron (III) citrate 0.5; magnesium sulfate 0.4; Agar-agar 15.0; pH=7.1± 0.2.

Preparation:

Suspend 80,5 g of the Salmonella Agar in 1 liter of demineralized water by heating in a boiling water bath. Incubate the medium plates, containing a pure bacterial culture, at 35-37°C for 24-48 hours.

1.2.3 GSP-Agar (*Merck)*

Pseudomonas/Aeromonas selective agar acc. to KIELWEIN (base) for microbiology.

Composition (g/Liter):

Sodium-L(+) glutamate 10,0; starch, soluble 20,0; potassium dihydrogen phosphate 2,0; magnesium sulfate 0,5; Phenol red 0,36; Agar-agar 12,0; pH=7,0 ± 0,2

Preparation:

Suspend 45 g of the GSP Agar in 1 liter of demineralized water and autoclave for 15 minutes at 121 °C. *Incubation time*: 72 hours at 28°C.

1.2.4 SIM Agar

SIM agar is used for the identification of bacteria which can utilize certain amino acids. It offers three tests in one: a). Indole test, b). H_2S-test, c). Motility test

Composition (g/Liter):

Casein-peptone 20.0; peptone from meat 20.0; Ammonium ferric (III) citrate 6.6; sodium thiosulfate 0.2; Agar-agar 0.2; pH=7.2 ± 0.2.

Preparation:

Solution should be autoclaved for 15 minutes at 121°C.

1.2.5 Simmons Citrate Agar

Simmons citrate agar is used for the identification of gram negative bacteria using their ability of citrate utilization as carbon and energy source.

Composition (g/Liter):

Magnesiumsulfate 0.2; Ammonium dihydrogen phosphate 0.2; Di-potassium hydrogen phosphate 0.8; Tri-sodium citrate x $2H_2O$ 2.28; sodium chloride 5.0; Bromthymolblue 0.08; Agar 15.0; pH=7.0 ± 0.2.

Preparation:

Prepared solution has to be autoclaved for 15 minutes at 121°C and than is ready to be aliquot into plates or tubes. Inoculate the medium with pure bacterial culture and incubate four days at 37°C.

1.2.6 Cytochrome Oxidase Test *(Fluka)*

Cytochrome oxidase test is used for the detection of microbial oxidase activity.

You have to mix together Oxidase Reagent A and Oxidase Reagent B according to Gaby-Hadley in 1:1 dilution and in drops tests the bacterial sample.

2. Bacterial Growth Media

2.1 Buffered Peptone Water

Peptone water as a growth medium for different microorganisms is also used for a base for carbohydrate fermentation studies. Peptone water with phosphate buffer system is commonly used for the recovery of bacteria after long exposition to environmental stress (change in the pH of the medium).

Composition (g/L):

Tryptone 10.0; sodium chloride 5.0; di-sodium hydrogenphosphate-dodecahydrate 9.0; potassium dihydrogenphosphate 1,5.

Preparation:

Dissolve all named components in demineralised water and autoclave for 10 minutes at 121 °C. (pH=7,2±0,2).

2.2 MRS Medium

For the growth of Lactobacillus and Leuconostoc strains (*Lactobacillus curvatus, Lactobacillus sakei, Leuconostoc mesenteroides*) MRS medium was used here. Bacteria (supplied from AGES) were incubated in this medium for 24 hours at 37 °C (200 ml bacterial culture).

Composition (g/L):

Casein peptone 10.0; meat extract 10.0; yeast extract 5,0 ; glucose 20.0; Tween 80 1.0; K_2HPO_4 2.0; Na-acetate 5.0; $(NH_4)_2$-citrate 2.0; $MgSO_4.7H_2O$ 0.20; $MnSO_4.H_2O$ 0.05; distilled water 1000 mL; pH=6.2-6.5 .

2.3 LM-Medium

LM-Medium is a liquid medium for bacterial growth and fermentation induction.

Composition (g/L):

Tryptone 10.0; yeast extract 10.0; Na_2HPO_4 x $2H_2O$ 0.4.

Preparation:

Dissolve all named components in demineralised water and autoclave for 10 minutes at 121 °C.

2.4 Ringer Solution

Ringer solution was used for dillutions of bacterial cultures for detection of bacterial counts.

Composition (g/L):

Sodium chloride 2.25; potassium chloride 0.105; calcium chloride x 6H2O 0.12; sodium bicarbonate 0.05; pH=7.0±0.2

Preparation:

Dissolve all above components in distillated water and autoclave for 15 minutes at 121 °C.

3. Gram Negative Bacteria for Homogenate Preparation

For the simulation of meat spoilage which takes places on the meat surface after first contact with bacteria, a couple of protocols for the meat homogenates and bacterial cocktails were developed. The bacteria used for the artificial meat infection were: *Serratia liquefaciens, Lactobacillus curvatus, Lactobacillus sakei, Leuconostoc mesenteroides, Brochothrix thermosphacta, Acinetobacter lwoffi, Pseudomonas ludensis, Pseudomonas fragi and Pseudomonas fluorescens, kindly provided by AGES (Austria Agency for Food Safty)*. Besides of these bacteria, *Proteus, Enterobacter and Salmonella* strains were also used.

	Preparation
Bacterial Cocktail A	Four gram negative bacteria (*Pseudomonas, Enterobacter, Proteus, Salmonella*), isolated from pork meat were mixed together 1:1 in pepton water with final bacterial concentration of 10^6 cfu/mm^3.
Bacterial Cocktail B	Nine gram negative bacteria (Serratia liquefaciens, Lactobacillus curvatus, *Lactobacillus sakei, Leuconostoc mesenteroides, Brochothrix thermosphacta, Acinetobacter lwoffi, Pseudomonas ludensis, Pseudomonas fragi and* Pseudomonas flourescens) were mixed in equal amount with final bacterial concentration of 10^6 cfu/mm^3.
Bacterial Cocktail C	Pseudomonas bacteria isolated from pork meat using GSP selective medium and cultivated in pepton water. Total bacterial count: 10^7 cfu/mm^3.

Table 1. Bacterial cocktails used for the meat homogenate preparation and sensor sensitivity tests.

3.1 Homogenate I and Homogenate II

Meat samples (pork steaks and pork cutlets) were supplied from the local supermarket (Billa). All experimental steps were performed under sterile conditions (in sterile bench by Bunsen burner). Meat samples were placed in Petri dishes and infected with Bacterial Cocktail B (3 ml of this bacterial mix for 300 g of meat) in bacterial concentration of 100 cfu/g. Infected meat samples for Homogenate I were incubated for 40 hours at 25°C, and for Homogenate II for 11 days at 4°C. Contaminated meat was transferred in 100 ml Ringer Solution into Stomacher Bags. Homogenisation of the samples was proceeded using the Stomacher® 400 Circulator, 1 min by 230 rpm. Thereafter, homogenized samples were frozen in 1 ml aliqoutes in liquid nitrogen and stored at -80 °C, to prevent further growth of bacteria. Meat bacteria in a such way prepared homogenate, and under abovestorage conditions are able to survive for about six months. As a precaution step, the total bacterial count using the Plate Count Agar was proceeded every eight weeks. There was no important variance in bacterial counts. Exposure of microorganisms to preservation treatments or environmental stresses which include refrigeration, freezing, heating, irradiation, desiccation, high salt, acid or alkali levels, nutrient limitation or treatment with disinfectants can cause different bacterial injuries. However, these meat bacteria which were isolated, stored and used for different homogenate preparations under above mentioned conditions, were fully intact after storage at -80°C and still able to produce lipolytic enzymes.

Figure 3. Stomacher® 400 Circulator
Source of the image:
www.biosci-intl.com/images/stomacr.jpg

3.2 Liquid Media for Lipase Production

Advantage of work with microorganisms as enzyme sources is the possibility of manipulating with their increased enzyme level due to enviromental or genetic changes. Bacteria have short fermentation cycles and that fact makes them attractive for different screening procedures [12]. Therefore it is very important to establish appropriate media conditions and parameters (temperature, pH, energy source) which are stimulative for bacterial growth and expected enzyme production. Of importance here are also inducers, substrates such as starch, glucose, lactose, phosphate (depending from bacterial strain and its fermentation process). Liquid medium (LM-Medium) which was used in this work for induction of bacteria for lipase production contained phosphate in form of Na_2HPO_4 x $2H_2O$. Phosphate is important inducer and activator of lipolytic enzymes such as aminopeptidase and phospholipase. The main goal was to find the right media which is able to simulate conditions at the meat surface and spoilage grade.

3.3 Bacterial Cocktail D

Bacterial cocktail D was prepared in Liquid Media (LM-Media) with phosphate as enzyme excretion inducer. The pork meat juice in volume of 1 ml (divided in 100 µl aliquotes) was spoiled after its incubation at 37°C for 5 hours, in a 37°C drying chamber. Aerobe gram negative bacterial strains: *Pseudomonas, Enterobacter, Proteus* and *Salmonella* were identified and isolated with help of selective media (GSP- and ÖNÖZ Salmonella-Agar). Bacterial colonies were picked with inoculating loop, according to their morphology (Table 1.), and inoculated in 3 ml liquid medium. Starter culture was incubated at 37°C for 24 hours under shaking at 180 rpm. On the next day, from the starter culture in 1:200 dilution 100 ml of main culture was prepared and bacteria were growing at 30°C for 16 hours, under shaking at 180 rpm. Bacterial cocktail (Bacterial Cocktail D) prepared in this way was aliquoted in 1 ml volume, frozen in liquid nitrogen and stored at -80 °C.

Preparation and storage of bacterial cultures along to this protocol showed the best results, because the bacterial growth was stopped and lipolytic enzymes protected. The experiment with glycerin as a storage additive also showed its protective role for the bacteria against environmental stress. However, glycerine prevented building of ice crystals and bacteria continued their growth and spent faster their nutrition supplies in growing media. That was the reason why all bacterial cultures which are presented in this work were stored without glycerine.

3.4 Homogenate III and Homogenate IV

Meat samples (pork cutlets) were supplied from the local supermarket (Spar). All experimental steps were performed under sterile conditions (in sterile bench by Bunsen burner). Meat samples (10g) were placed in Petri dishes and infected with Bacterial Cocktail D in bacterial concentration of 1000 cfu/g. Infected meat samples for Homogenate III were incubated for 96 hours at 4 °C, and for Homogenate IV for 48 hours at 15°C. Contaminated meat was transferred in 90 ml Liquid Media in Stomacher Bags. Homogenisation of the samples was proceeded using the Stomacher® 400 Circulator, 1 min by 230 rpm. Thereafter, homogenized samples had been frozen in 1 ml aliqoutes in liquid nitrogen and stored at -80 °C, to prevent further growth of bacteria.

3.5 Milk Homogenate M1 and M2

Pasteurised milk sample of 50 ml was incubated for four days at 4 °C and room temperature. Total bacterial count was done with Plate Count Agar. Spoiled milk was shortly vortexed and stored in 1 ml aliquotes at -20 °C.

3.6 Vegetable Homogenate G1 and G2

Vegetable mixture (Ajvar) without preserving agent (10 g) was incubated for four days at 4 °C and room temperature. Thereafter, the samples were centrifuged three times at 3000 rpm and each time only the supernatant was used for further centrifugation steps. Aliquotes (100 µl) were stored at -20 °C.

4. Bacterial Cultures Preparation for Higher Lipase Yield

4.1 Bacterial Cultures HIIA and HIIB

Bacterial samples presented here, were prepared according to [13] in a way to gain the highest lipase yield level. The main bacterial culture (100 ml) containing the bacteria from homogenate II starter culture was incubated for 6 hours (HIIA) and 24 hours (HIIB) in a water bath at 15 °C. After this period of bacterial growth, the cultures had the total bacterial counts of ~10^7 CFU/mm^3 for HIIA and ~10^8 CFU/mm^3 for HIIB. Afterwards, the bacterial cultures were frozen in liquid nitrogen and stored in 100 µl aliquotes at -80°C.

4.2 Bacterial Cultures HII-1 and HII-2

Bacterial cultures HII-1 and HII-2 were also growing in LM-Media (liquid media) for 4 days in 15°C water bath. Homogenate II bacteria were used for the preparation of starter culture (or as a start culture) and thereafter for the main bacterial culture in 1:200 dilutions. The culture samples (1 ml aliquots) were centrifuged for 5 minutes in a table top centrifuge at 4 °C. The supernatant with maximal enzyme concentration was frozen in liquid nitrogen and stored at -80 °C. Thereafter, the samples were used for sensor sensitivity tests.

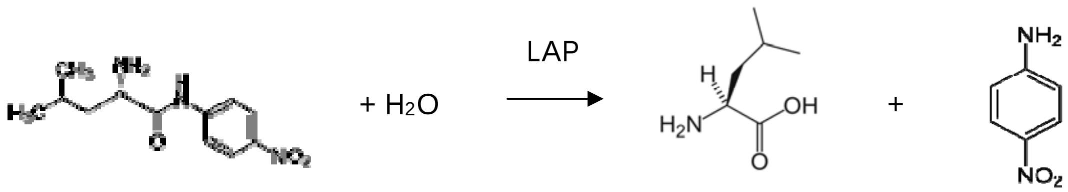
4.3 Bacterial Cultures PS1 and PS2

For the preparation of these bacterial cultures the previos protocol (for HIIA and HIIB) was used. Only differences are the starter bacterial cultures which in this case were prepared from two Pseudomonas colonies (PS1 and PS2), isolated from the spoiled pork meat juice. The aliquots (100 µl) were also frozen in liquid nitrogen and stored at -80°C.

5. Screening for Enzyme activity

5.1 Quantitative

5.1.1 Leucine Aminopeptidase Assay *(Sigma)*



L- leucine p- nitroanilide L-leucine p-nitroaniline

LAP = Leucine Aminopeptidase, Microsomal

Conditions: T = 37 °C, pH = 7.2, A_{405nm}, Light path = 1 cm
Method: Continuous Spectrophotometric Rate Determination

Reagents:

A. 60 mM Phosphate Buffer, pH 7.2 at 37 °C

(Prepare 100 ml in deionized water using potassium phosphate, monobasic, anhydrous. Adjust to pH 7.2 at 37 °C with 1 M KOH.)

B. 1.66 mM L-Leucine p-Nitroanilide Solution (L-Leu_NA)

(Prepare 30 ml in Reagent A using L-leucine p-nitroanilide hydrochloride. Prepare fresh!)

C. 10 mM TrisHCl Buffer with 1 mM $MgCl_2$, pH 8.0 at 37 °C (Activation Buffer)

(Prepare 20 ml deionized water using Trizma Base. Adjust to pH 8.0 at 37 °C with 1 M HCl and then add $MgCl_2.6H_2O$).

D. Leucine Aminopeptidase Enzyme Solution, Non-Activated (Enz-Non Act)

(Immediately before use, prepare a solution containing 0.3 unit/ml of leucine aminopeptidase in cold deionized water.)

E. Leucine Aminopeptidase Enzyme Solution, Acivated (Enz-Act)

(Immediately before use, prepare a solution containing 0.1 unit/ml of leucine aminopeptidase in cold Reagent C. Incubate at 37 °C for 15 minutes to activate.

Experimental procedure:

Pipette (in millilitres) the following reagents into suitable cuvettes:

	Non-Acivated		**Activated**	
	Test	Blank	Test	Blank
Reagent B (L-Leu-NA)	2.00	2.00	2.00	2.00
Equilibrate to 37 °C. Monitor A405nm until constant, using a suitably thermostated spectrophotometer. Then add:				
Deionized Water		0.10		
Reagent C (Activation Buffer)				0.10
Reagent D (Enz Non Act)	0.10		0.10	
Reagent E (Enz Act)				

Immediately mix by inversing the cuvette and record the increase in A_{405nm} for approximately 5 minutes. Obtain the ΔA_{405nm}/minute using the maximum linear rate for both the Test and Blank.

Calculations:

With help of calibration line (increased leucine - aminopeptidase activity).

Generation of Calibration Line

The aim was to generate a calibration line with a stock of leucine aminopeptidase (10 U/mg). Dilutions of enzyme solutions were made with deionized water (for non-activated) and reagent C (for activated form).

The following activities of leucine aminopeptidase were used for the activated case: 10, 25, 50, 60, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mU/mg. For the non-activated, the activities were the following: 10, 15, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mU/mg.

Unit Definition:

One unit will hydrolyze 1.0 µmole of L-leucine p-nitroanilide to L-leucine and p-nitroaniline per minute at pH 7.2 at 37 °C.

Final Assay Concentration:

Non-Activated: In a 2.10 ml reaction mix, the final concentrations are 58 mM potassium phosphate, 1.61 mM L-leucine p- nitroanilide and 0.03 unit leucine aminopeptidase.

Activated: In a 2.10 ml reaction mix, the final concentrations are 58 mM potassium phosphate, 1.61 mM L-leucine p- nitroanilide, 0.32 mM Tris HCl, 0.03 mM $MgCl_2$ and 0.01 unit leucine aminopeptidase.

5.2 Qualitative

5.2.1 Trybutyrin Agar Base *(Acila Sarl)*

Trybutyrin Agar Base is a solid culture medium for testing the lipolytic capacity of microorganisms.

Preparation:

Dissolve 24 g of trybutyrin agar base powder in 1 l destilled water and heat (50-75 °C) to dissolve completely. Thereafter, add 10 ml of 98% glycerine-trybutyrate *(Sigma-Aldrich)* and sterilize by autoclaving at 121 °C for 10 minutes, mix and pure very hot into plates.

6. Gravure Printing

Gravure printing is the technique for printing processes in which the incision of the image has to be printed into a surface (printing plate). These incisions are mainly created by etching or engraving, thus producing lots of small recessed cells that act as tiny wells. Their depth and size control the amount and homogeneity of ink or lacquer that gets transferred to the substrate (such as paper or other materials, e.g. plastic-foils). The smoother the pattern of incisions, the better is the intensity of the printed color and brilliance [6].

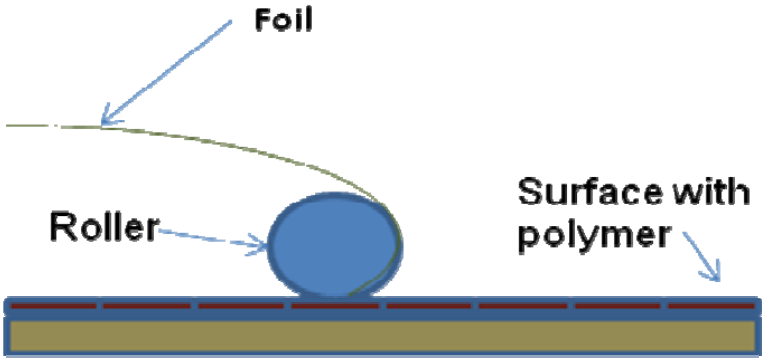


Figure 4. Schematic illustration of the gravure printing process: a roller presses the foil onto the surface of printing plate containing polymer solution.

The substrate gets sandwiched between the impression roller and the printing plate. This is where the ink is transferred from the recessed cells to the foil. The impression roller applies force, pressing the foil sheet onto the gravure cylinder, ensuring even and maximum coverage of the ink. The printed foil was dried for 10 minutes at 80°C, washed for 10 minutes in demineralized water and again dried at 80 °C for 10 minutes. Through these washing and drying steps all salt and solvent rests were removed.

Figure 5. Gravure Printer (K Printing Proofer 628 by Erichsen)

7. Sputter Coating

Sputter coating is an electronic device for target bombarding with fast heavy particles. Sputtering is the term for processes where atoms are ejected from a solid target material into the gas phase [14]. The atoms have statistically defined energy and direction in space. This observation leads to an omni-directional deposition of the sputtered atoms forming coatings of the original cathode material on the surface of the work chamber and certainly on the specimen that is placed in a definite distance below the target material. The possible layer thickness of sputter coating methods lies between just a few nanometers and a cohesive surface of optional gauges.

Commonly, sputter coating is used for thin-film deposition, as well as analytical techniques, for example in SIMS (Secondary Ion Mass Spectroscopy), where the target sample is sputtered and afterwards its concentration and identity can be measured using MS (Mass Spectroscopy). In the case of this work the method of choice was sputter coating to produce a gold-nanoparticle-thin-film of approximately 5 nm thicknesses (Results and Discussion, Fig.12). In general there are several elements that can be exerted as target material, for example palladium, silver, copper and tin. These metals are deposited as atoms that assemble to islands that are usually rather flat and asymmetric.

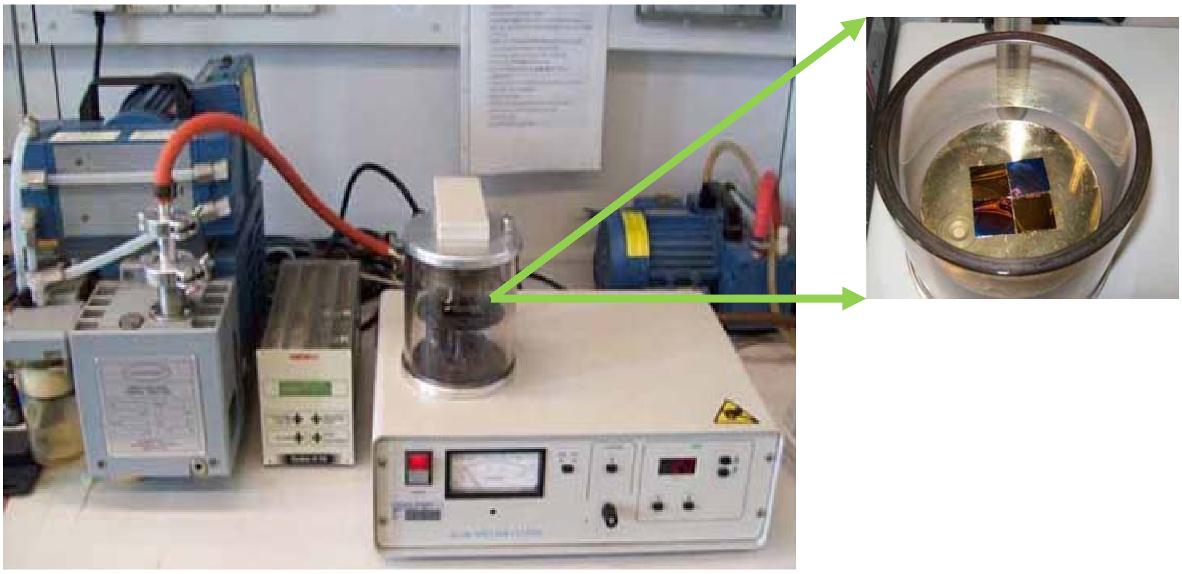


Figure 6. Agar Sputter Coater

The most common arrangement for a D.C. sputter coater is defining the (gold) cathode as the target material to be sputtered, and to locate the sensors to be coated on the anode. The desired operating pressure of 0.08 mbar is obtained by a turbo molecular pump, with an ultra-pure inert gas, such as argon, admitted to the chamber by a fine control valve.
The argon performs two tasks. First of all it is used to rinse the chamber in order to displace the present air. The regulation of argon concentration within the chamber enables the adjustment of a distinct pressure at which the plasma can be ignited.

Furthermore, by including argon in a cathode gas tube, the free ions and electrons are attracted to opposite electrodes and a small current is produced.

And as the voltage is increased, some ionization is induced due to collisions of electrons with gas atoms. A further raise in supply voltage concentrates the arisen glow in a cathode spot and arc discharge appears which means that free ions and electrons are attracted to opposite electrodes.

Once the condition for a sustained discharge is met, the tube exhibits the characteristic glow discharge, so called because of the associated luminous glow.

For a self sustaining discharge the regeneration of electrons by positive ion bombardment of the cathode is required.

Secondary electrons are produced and ionization enhanced, but the most important consequence is the erosion of the cathode material and the emission of gold atoms, which are distributed in the whole reaction chamber and impinge on the specimen's surface – as intended. This process is enhanced in sputter coaters for use in Scanning Electron Microscopy (SEM) where one objective is to provide an electrically conductive thin film representative of the surface topography of the specimen [15].

8. Sensor Setup

The idea was to create a simple and cheap biosensor providing high sensitivity and selectivity for determination of bacterial infection. The current tendency to carry out field monitoring has driven the development of biosensors as new analytical tools able to provide fast, reliable and sensitive measurements with lower cost. These biosensors for the moment do not compete with official analytical methods, but they can be used both – by regulatory authorities and by industry to provide enough information for routine testing and screening of samples [16].

Here is presented a simple two-layer biosensor with poly glycolic lactic acid as a distance layer (Fig. 7).

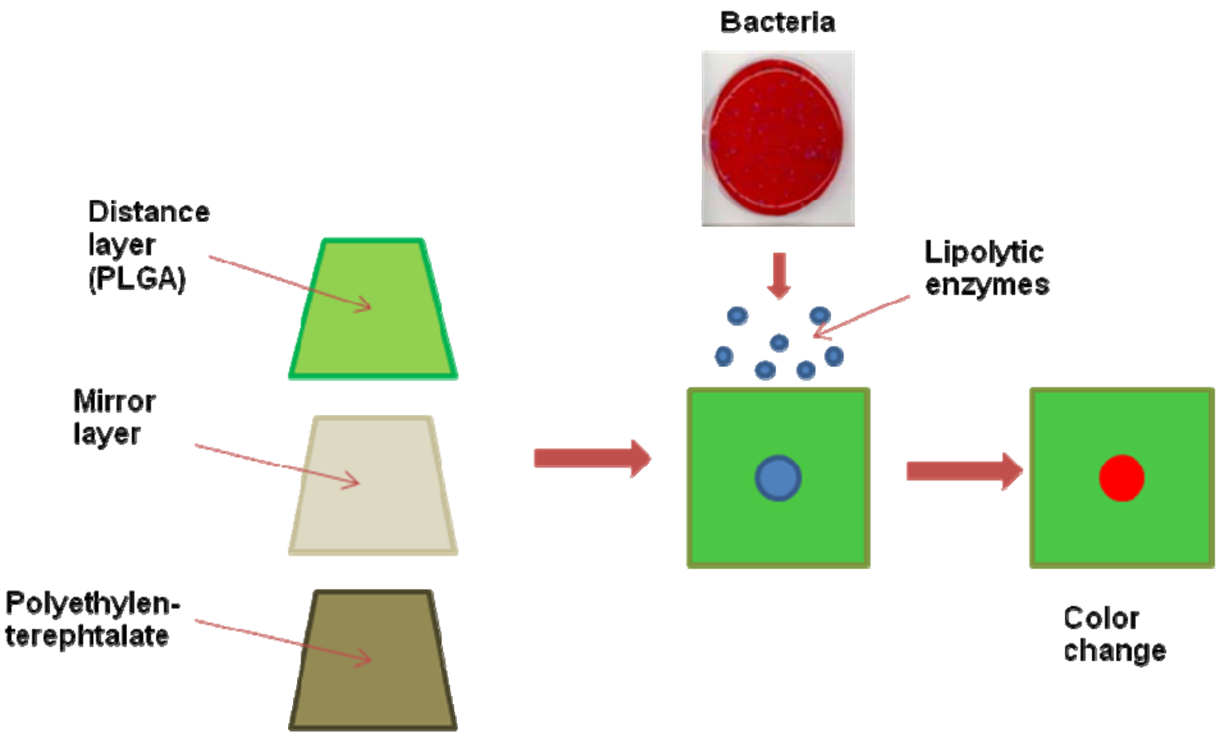


Figure 7. Sensor setup with PLGA as distance layer/second layer.

8.1 Mirror Layer

The mirror layer is of great importance for the sensor setup. It has to be very stable and to contain a smooth surface able to interact with the nanoparticles at a definite distance that results in anomalous absorption. As a mirror layer for already described sensor setup (Fig. 7) Inconnel was used. Inconnel is a nickel-chromium alloy with good oxidation resistance and often used in food processing. In the majority of the cases the prefabricated Inconnel and gold coated PET-foils were used. Drawing a comparison between the Inconel, gold and silver mirrors requires the testing of the stability of the associated sensors (complete setup) towards high temperature and salt concentration.

8.2 PLGA Distance Layer

Since it is the task of the distance layer (second layer in two-layer sensor, Fig. 7) to be degraded by microorganisms resposible for food spoilage, it must have the ability to react with various microbial and cellular lipolytic enzymes. The tickness of distance layer is also very important [26]. For this purpose the use of PLGA [poly (lactic-coglycolic acid)] as a biodegradable polymer showed extraordinary results.

8.3 Poly (lactide-co-glycolide)

Various biodegradable polymers are already well known for the production of nanoparticles aimed at drug and gene delivery applications [19]. Poly (D, L lactide-co-glycolide) (PLGA) and poly (lactic acid) (PLA)-both of which are FDA-approved biocompatible polymers-have been the most extensively studied [20, 21]. Recently, were reviewed the PLGA nanoparticle preparation techniques, and reported an emulsion-solvent evaporation technique to be the most widely used method to formulate PLA and PLGA nanoparticles, while poly(vinyl alcohol) (PVA) is the most commonly used stabilizer [20]. Labhasetwar and co-workers [22-25] made several reports on aspects of PLGA nanoparticles for gene delivery, characterization of nanoparticles uptake by endothelial cells, size dependency of nanoparticle-mediated gene transfection [23], effects on nanoparticle properties and their cellular uptake associated with the residual PVA [24], and rapid endolysosomal escape of the PLGA nanoparticles [25]. They followed the standard emulsion-solvent evaporation technique, which resulted in particles with a heterogeneous size distribution. Moreover, they investigated the relative transfectivity of the smaller and the larger-sized particles in cell cultures [23]. Those particles which passed through a membrane of pore size 100 nm were designated as smaller particles, whereas those retained on the membrane were deemed to be larger-sized nanoparticles (202 ± 9 nm) [17].

9. Solvents for Polymer Dilution

Dependent on the PLGA characteristics such as chain length and solubility it was very important to find an appropriate solvent. The best ones for the necessary requirements as costs and toxicity were ethyl acetate and 2,2,2-trifluoroethanol.

10. Crosslinker

The role of the crosslinker for the polymer is assumed by Desmodur (diphenylmethanediisocyanate or triphenylmethane- 4, 4‘, 4‘‘- triisocyanate) from BAYER. The crosslinker strongly influences the viscosity and composition of the polymer solution and thus, the thickness of the polymer layer.

11. Sensor Sensitivity Test

In order to test the potential of various sensors for changing their colour, they were investigated regarding their reaction to various dilutions of decomposed meat, milk or vegetable homogenates and the bacterial cocktails. Approximately 2 µl of the sample was pipetted onto the sensor surface and incubated in a humid chamber for 4, 6 and 16 hours at 4°C, room temperature (~ 25 °C) and 37 °C. After these incubation times, the sensors were washed with double-distilled water and then dried under an intensive air-stream. Due to the fact that this sensor’s application domain is the observation of a colour change with the naked eye, each signal must be perceived easily by just looking at it.

The examined sensors were checked optically and scanned. Scanning was somewhat difficult, because of the peculiarity of the sensors, their ability to shift the colour depending on the visual angle. The use of a scatter filter, in this case Para film, ensured reproducibility and nice result’s presentation.

Results and Discussion

1. Isolation, Identification and Total Count of the Food (Meat) Bacteria

1.1 Total Count of the Food (Meat) Bacteria

To determine an entire number of bacteria which are growing on the spoilt meat, we used the Plate Count Agar. This is a microbiological nonselective growth medium for the monitoring of total bacterial growth of the sample.

Dilutions	Pseudomonas	Proteus	Salmonella	Enterobacter
undiluted	b	b	b	b
1:10	b	b	b	b
1:100	b	b	b	b
1:1000	b	b	b	$4{,}6x10^6$ cfu/mm^3
1:10 000	b	$3{,}22x10^7$ cfu/mm^3	b	$8{,}8x10^6$ cfu/mm^3
1:100 000	$5{,}82x10^8$ cfu/mm^3	$4{,}6x10^7$ cfu/mm^3	$5{,}92x10^8$ cfu/mm^3	$1x10^7$ cfu/mm^3
1:1 000 000	$1{,}4x10^9$ cfu/mm^3	$8x10^7$ cfu/mm^3	$192x10^9$ cfu/mm^3	$3x10^7$ cfu/mm^3

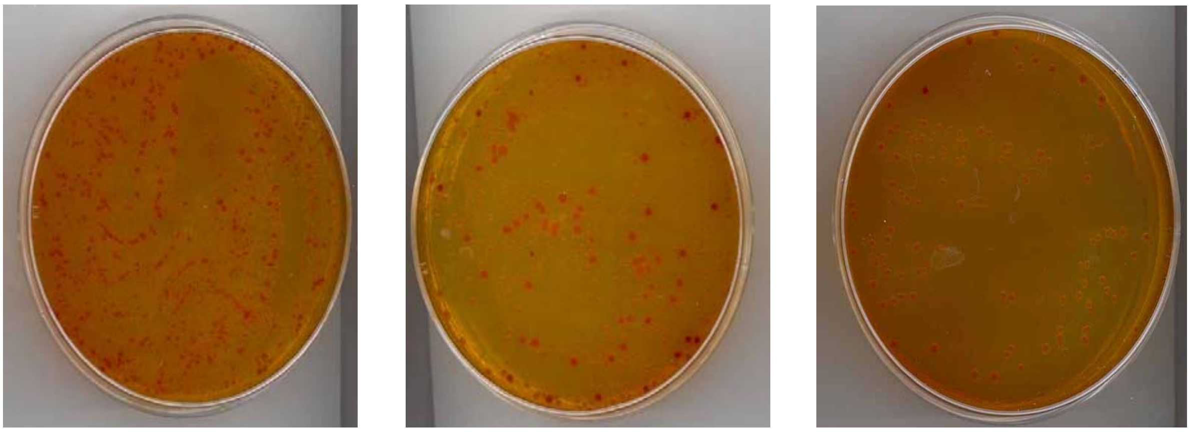
Table 2. Total bacterial count of the meat bacteria. Four bacterial cultures of *Pseudomonas, Proteus, Salmonella and Enterobacter* were inoculated in pepton water (1:200 dilutions) and incubated for 24 hours at 37°C.

1.2 Identification of Pseudomonas, Enterobacter, Proteus and Salmonella

ÖNÖZ Agar is a selective medium for the isolation and identification of *Salmonella* and some other *Enterobacteriacaea.*

MICROORGANISMS	APPEARANCE OF COLONIES
Pseudomonas	Glossy, dirty yellow to greenish; Culture medium is yellow
Enterobacter	Large, mucoid, bluish or reddish, slight precipitation ring around the colonies
Proteus	Rust-colored, culture medium surrounding the colonies of same color, if growth is too dense, dark brown to black
Salmonella	Yellow, medium size; 1st day: black dots start to develop on the yellow colonies; 2nd day: black dots clearly visible on the yellow colonies; culture medium surrounding the colonies is yellowish

Table 3. Typical colonial morphology of the bacteria isolated with ÖNÖZ agar.



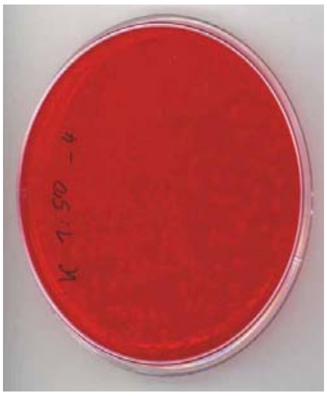
1:100 dilution 1:1000 dilution 1:10 000 dilution

Figure 8. Isolated aerobe gram negative bacteria from a spoilt pork meat juice (incubated for 5 hours at 37 °C). Dilutions were prepared in Ringer Solution (pH 8.2) and tested on the ÖNÖZ agar plates. Based on Table 3 it was possible to identify four of the bacteria which are responsible for the spoilage of the meat: *Pseudomonas, Enterobacter*, *Proteus and Salmonella*.

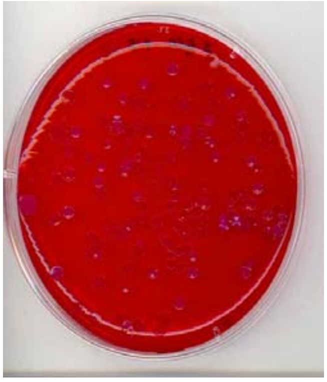
1.3 Detection of Pseudomonads

Pseudomonas/Aeromonas selective agar acc. to KIELWEIN (base) for microbiology: This is a glutamate starch phenol-red agar with glutamate and starch as its only nutrients. Starch can be degraded by *Aeromonas* producing the acid (color change of phenol-red to the yellow, but not by *Pseudomonas*). For the improvement of its selectivity it is possible to add some selective inhibitors such as penicillin or antymycotic pimaricin.

Cutlet 1:50



Steak 1:50



Cutlet 1:50 (photo from the top)

Figure 9. GSP agar plates with *Pseudomonas* bacteria isolated from a different meat homogenates. *Pseudomonas* appears as blue-violet large colonies with diameter of 2-3 mm, souranded by a red-violet zone.

1.4 Pseudomonas are the main aerobic meat spoilage psychotrops

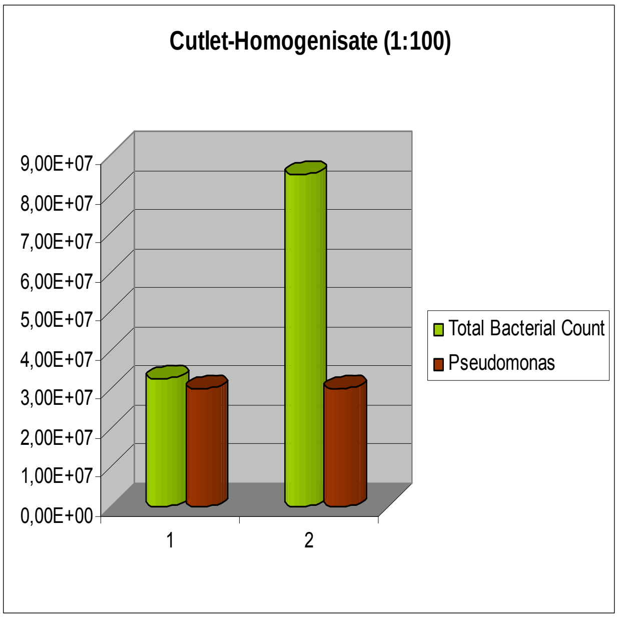


Diagram 1a.

Correlation between *Pseudomonas* species and the total bacerial count. Investigated sample was a pork cutlet homogenate diluted 1:100 in Ringer Solution.

Pseudomonas are gram negative, strict aerobes, oxidase positive bacterial strains which are present in highest level of all bacteria in spoiled meat. *Pseudomonas* genus, with metabolic diversity, belongs to the larger family of Pseudomonads. These bacterial strains have been found in all sorts of meat (pork, fish, beef, chicken). There are three Pseudomonas species identified in pork meat so far: *Pseudomonas ludensis, Pseudomonas fragii and Pseudomonas flourescens.* As a growth substrate they use glucose-6-P, pyruvate, gluconat, lactic acid, gluconat-6-P, glycerin and tributyrate.

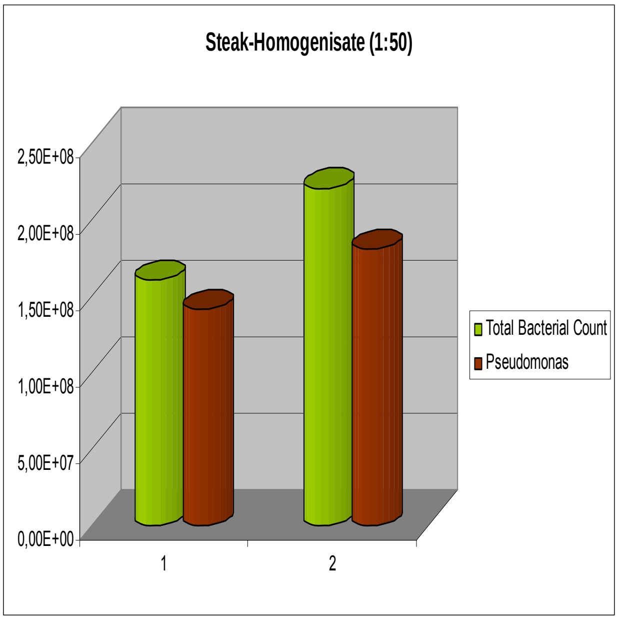


Diagram 1b.

Correlation between *Pseudomonas* species and the total bacerial count. Investigated sample was a pork steak homogenate diluted 1:50 in Ringer Solution.

For the utilisation of proteins and fatty acids as an energy source, *Pseudomonas* are forced to produce extracellular enzymes (lipases, proteases and aminopeptidase).

These enzymes are able to degrade these components and make them suitable for the uptake into the cell. These two diagrams are showing a comparison of total bacterial count (tested with Plate Count Agar) and *Pseudomonas* count (tested with GSP Agar), showing the high percentage of *Pseudomonas* in whole bacterial growth.

1.5 Identification of Gram Negative Bacteria

SIM agar is used for the determination of microorganisms which can metabolize certain amino acids. It offers three tests in one for the testing of gram negative bacteria (growth of the gram positive bacteria is inhibited):

(i) *<u>Indole test</u>*

Tryptophan (aromatic amino acid) has a ring form with an amine group and indole. Positive result is possible only if the bacteria can split this amino group and the indole.

(ii) *H$_2$S (Hydrogen Sulfide)-Test*

If the bacteria can metabolize hydrogen sulfide the agar will change its color to black. Kovac´s reagent reacts with indole and forms a bright red ring at the surface of the SIM Agar Tube. It comes to split of indole from the tryptophan molecule by tryptophanases.

(iii) *Motility Test*

Motility positive bacteria grow through the medium from the stab and motility negative bacteria grow only on the stabbed place.

	Proteus	Salmonella	Pseudomonas	Enterobacter
Motility	+	+	+	+
Indol	+/-	-	-	-
H_2S	+	+	-	-

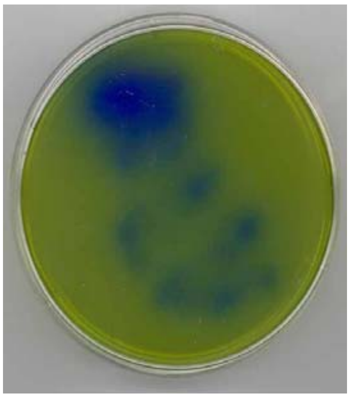
Table 4. Testing of bacterial motility, ability to build indole and hydrogen sulfide, using SIM agar tubes.

1.6 Evidence for Citrate Utilisation

Simmons citrate agar is a synthetic test agar for the identification of gram negative bacteria using their ability of citrate utilization; citrate as a sole carbon source and ammonium ions as a sole nitrogen source.

Pseudomonas

Proteus



Enterobacter

Salmonella

Figure 10. Simmons citrate agar for the evidence of citrate utilization by *Pseudomonas, Proteus, Enterobacter* and *Salmonella*. Seeded bacteria were incubated for four days at 37°C. Formation of ammonia led to alkalization of the medium and turning the color of bromthymolblue from green to blue.

	Proteus	Salmonella	Pseudomonas	Enterobacter
Citrate	+	+	+	+

Table 5. Summerized results of citrate utilisation for the four meat bacteria.

1.7 Cytochrome Oxidase

Cytochrome oxidase test is used to determine the ability of bacteria to produce this cytochrome c converting enzyme. For the detection of oxidase activity in selected microorganisms, we used Oxidase Reagent A and Reagent B according to Gaby-Hadley. Mix two reagents 1:1. By only 2-3 drops of the mixture onto growing bacteria sample (prepared before and incubated for 24 hours at 30°C) it is possible to test if the bacteria are oxidase positive or negative.

Pseudomonas (oxidase positive)

Salmonella (oxidase negative)

Figure 11. Determination of cytochrome c by using an oxidase test according to Gabby Hadley.

	Proteus	Salmonella	Pseudomonas	Enterobacter
Oxidase	+	-	-	-

Table 6. Summary of the oxidase activity results for *Pseudomonas, Proteus, Enterobacter and Salmonella.*

2. Bacterial Counts

2.1 Bacterial Counts for Homogenate I and Homogenate II

PORK STEAK	
Dilutions	**CFU/mm^3**
1:100 000	$5,3x10^7$
1:1 000 000	$4,5x10^7$
1:10 000 000	a
1:100 000 000	a
1:1 000 000 000	a
PORK CUTLET	
Dilutions	**CFU/mm^3**
1:100 000	$8,5x10^7$
1:1 000 000	$5x10^7$
1:10 000 000	a
1:100 000 000	a
1:1 000 000 000	a

Table 7a. Total bacterial count of the bacteria in Homogenate I. Incubation time: 40 h by 25°C.

PORK STEAK	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	b
1:10 000 000	$1,41x10^8$
1:100 000 000	$2,2x10^8$
1:1 000 000 000	a
PORK CUTLET	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	$3,27x10^7$
1:10 000 000	$8,9x10^7$
1:100 000 000	$8,5x10^7$
1:1 000 000 000	a

Table 7b. Total bacterial count of the bacteria in Homogenate I. Incubation time: 11 days by 4°C.

For the sensor sensitivity test, both homogenates were used in their stock bacterial count (10^7-10^8 cfu/g) and in dilution of 10^6 cfu/mm^3. All dilutions were performed with Ringer Solution (pH=8,2).

2.2 Bacterial Counts for Homogenate III and Homogenate IV

Homogenate III	
Dilutions	**CFU/mm^3**
1:1 000	$1,31x10^6$
1:10 000	$6,8x10^6$
1:100 000	$2,75x10^7$

Table 8a. Bacterial counts in Homogenate III

Homogenate IV	
Dilutions	**CFU/mm^3**
1:1 000	b
1:10 000	$2,68x10^7$
1:100 000	$6,5x10^7$

Table 8b. Bacterial counts in Homogenate IV

2.3 Bacterial Counts for HIIA and HIIB

Bacterial Culture HIIA	
Dilutions	**CFU/g**
1:1 000	b
1:10 000	$2,72x10^7$
1:100 000	$5,75x10^7$
1:1 000 000	a
1:10 000 000	a
1:100 000 000	a
1:1 000 000 000	a

Table 9a. Bacterial counts in

Bacterial Culture HIIA

Bacterial Culture HIIB	
Dilutions	**CFU/g**
1:100 000	$4,13x10^8$
1:1 000 000	$1,09x10^9$
1:10 000 000	$1,3x10^9$
1:100 000 000	a
1:1 000 000 000	a

Table 9b. Bacterial counts in

Bacterial Culture HIIB

2.4 Bacterial Counts for HII-1 and HII-2

Bacterial Culture HII-1	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	b
1:10 000 000	1,35x10^{10}
1:100 000 000	2,95 x10^{10}
1:1 000 000 000	2,5x10^9

Table 10a. Bacterial counts in

Bacterial Culture HII-1

Bacterial Culture HII-2	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	b
1:10 000 000	1,62x10^{10}
1:100 000 000	3,4 x10^{10}
1:1 000 000 000	4,5x10^9

Table 10b. Bacterial counts in

Bacterial Culture HII-2

2.5 Bacterial Counts for PS1 and PS2

Bacterial Culture PS1	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	6,64x10^9
1:10 000 000	2,24x10^{10}
1:100 000 000	1,3 x10^{10}
1:1 000 000 000	1,5x10^9

Table 11a. Bacterial counts in

Bacterial Culture PS1

Bacterial Culture PS2	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	b
1:10 000 000	8,8x10^9
1:100 000 000	9 x10^9
1:1 000 000 000	a

Table 11b. Bacterial counts in

Bacterial Culture PS2

2.6 Bacterial Counts for M1, M2, G1 and G2

Dilutions	Milk M1	Milk M2	Vegetable G1	Vegetable G2
1 : 100	2,5 x 10^3 cfu/mm^3	5,6 x 10^4 cfu/mm^3	1,2 x 10^3 cfu/mm^3	6,3 x 10^4 cfu/mm^3
1 : 10 00	a	a	a	a
1: 100 00	a	a	a	a

Table 12. Total bacterial counts of milk homogenate M1 and M2 and vegetable homogenate G1 and G2.

2.7 Total Bacterial Count in Bacterial Cocktail A

Pseudomonas	
Dilutions	**CFU/mm^3**
1:100 000	5,23x10^8
1:1 000 000	6,65x10^8
1:10 000 000	7,5x10^8
1:100 000 000	a
1:1 000 000 000	a

Table 13a. Bacterial counts of Pseudomonas in peptone water.

Enterobacter	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	1,57x10^9
1:10 000 000	1,75x10^9
1:100 000 000	a
1:1 000 000 000	a

Table 13b. Bacterial counts of Enterobacter in peptone water.

Proteus	
Dilutions	**CFU/mm^3**
1:100 000	$4{,}7x10^8$
1:1 000 000	$6{,}15x10^8$
1:10 000 000	$7{,}65x10^8$
1:100 000 000	a
1:1 000 000 000	a

Table 13c. Bacterial counts of Proteus in peptone water.

Salmonella	
Dilutions	**CFU/mm^3**
1:100 000	$5{,}8\ x10^8$
1:1 000 000	$5{,}65x10^8$
1:10 000 000	$1{,}45x10^8$
1:100 000 000	a
1:1 000 000 000	a

Table 13d. Bacterial counts of Salmonella in peptone water.

2.8 Total Bacterial Count in Bacterial Cocktail B

Bacterial Cocktail B	
Strains	**CFU/mm^3**
Serratia liquefaciens	$5{,}07\ x10^8$
Lactobacillus curvatus	$9x10^7$
Lactobacillus sakei	$6{,}77x10^7$
Leuconostoc mesenteroides	$3{,}6x10^7$
Brochothrix thermospacta	$1{,}9x10^7$
Acinetobacter lwoffi	$2{,}01x10^8$
Pseudomonas lundensis	$2{,}25x10^8$
Pseudomonas fragi	$9{,}05x10^7$
Pseudomonas flourescens	$1{,}62x10^8$

Table 14. Bacterial counts of gram negative bacteria, provided by AGES.

2.9 Total Bacterial Count in Bacterial Cocktail C

Bacterial Cocktail C	
Dilutions	**CFU/mm^3**
1:1 000	b
1:10 000	2,62x10^7
1:100 000	2,6x10^7
1:1 000 000	2 x10^7
1:10 000 000	a
1:100 000 000	a
1:1 000 000 000	a

Table 15. Bacterial count of Pseudomonas culture, prepared in peptone water.

2.10 Total Bacterial Count in Bacterial Cocktail D

Pseudomonas	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	b
1:10 000 000	7,3x10^9
1:100 000 000	a
1:1 000 000 000	a

Table 16a. Bacterial counts of Pseudomonas in LM-Media.

Enterobacter	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	2,7x10^9
1:10 000 000	8,35x10^9
1:100 000 000	1,45x10^{10}
1:1 000 000 000	a

Table 16b. Bacterial counts of Enterobacter in LM-Media

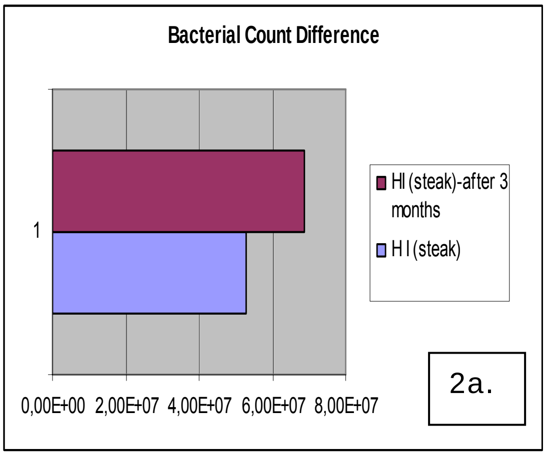
Proteus	
Dilutions	**CFU/mm^3**
1:100 000	$3,69x10^8$
1:1 000 000	$7,8x10^8$
1:10 000 000	$1,4x10^9$
1:100 000 000	a
1:1 000 000 000	a

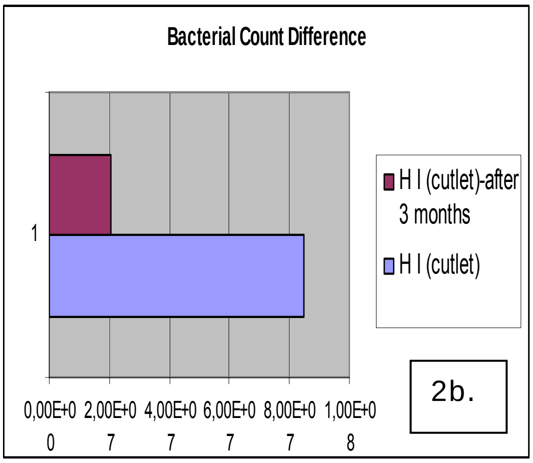
Table 16c. Bacterial counts of Proteus in LM-Media.

Salmonella	
Dilutions	**CFU/mm^3**
1:100 000	b
1:1 000 000	b
1:10 000 000	$1,02x10^{10}$
1:100 000 000	$1,55x10^{10}$
1:1 000 000 000	a

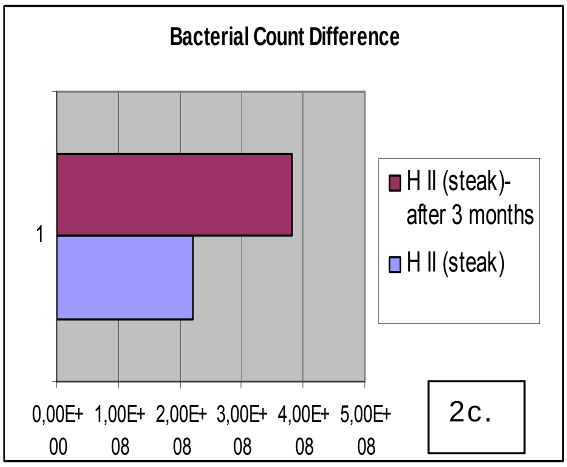
Table 16d. Bacterial counts of Salmonella in LM-Media.

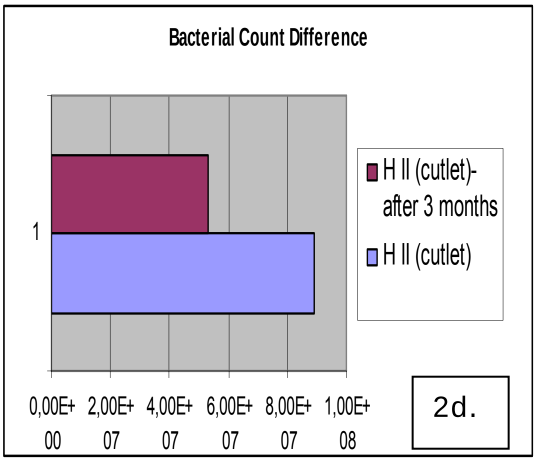
3. Difference of Bacterial Counts in Correlation with Storage Conditions





Diagrams 2a. and 2b. Comparison of bacterial growth in Homogenate I after 3 months storage at -80°C.





Diagramms 2c. and 2d. Comparison of bacterial growth in Homogenate II after 3 months of storage at -80°C.

4. Substrate Addition

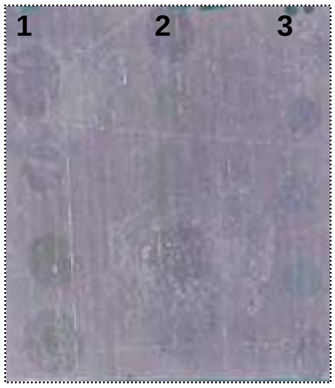
All original solutions were prepared in 2ml volume. PLGA was dissolved in triflourethanol or in ethylacetate under addition of substrates or respectively in before prepared stock solution of substrates in one of these two solutions. Results for sensor sensitivity are presented with: (+):very strong signal and change of color; (+/-):signal is present but without observable color change; (-): there is no signal at all.

4.1 Addition of Tryptone and Glucose

PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C
21%	$5x10^{-8}$ %	- / +/- / +
21%	$5x10^{-6}$ %	- / +/- / +
21%	$5x10^{-3}$ %	- / +/- / +
23%	$5x10^{-8}$ %	- / - / +
23%	$5x10^{-6}$ %	- / - / +
23%	$5x10^{-3}$ %	- / - / +
25%	$5x10^{-8}$ %	- / - / +
25%	$5x10^{-6}$ %	- / - / +
25%	$5x10^{-3}$ %	- / - / +

Table 17a. Experiment with tryptone and glucose dissolved in triflourethanol as stock solution (50 mg tryptone and 10 mg glucose in 10 ml triflourethanol). PLGA was added later and dissolved at room temperature.

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10^8 cfu/mm^3	6. Homogenate I 10^5 cfu/mm^3	11. Enzyme Mix* 20 mg/mm^3
2. Bacterial Cocktail A 10^6 cfu/mm^3	7. Homogenate II 10^8 cfu/mm^3	12. Enzyme Mix* 15 mg/mm^3
3. Bacterial Cocktail A 10^5 cfu/mm^3	8. Homogenate II 10^6 cfu/mm^3	13. Enzyme Mix* 10 mg/mm^3
4. Homogenate I 10^8 cfu/mm^3	9. Homogenate I 10^5 cfu/mm^3	14. Enzyme Mix* 5 mg/mm^3
5. Homogenate I 10^6 cfu/mm^3	10. Bacterial Cocktail C 10^7 cfu/mm^3	15. Ringer Solution



1
2
3
4
5

After 16h at room tepmeratur

Table 17b. Pipetting scheme. ***** Equal amount of Proteinase K, Chymotrypsine and Trypsine.

PLGA Concentration	Desmodur (triisocyanat)	Sensor Sensitivity 4°C/RT/37°C	PLGA Concentration	Desmodur (triisocyanat)	Sensor Sensitivity 4°C/RT/37°C	PLGA Concentration	Desmodur (triisocyanat)	Sensor Sensitivity 4°C/RT/37°C
21%	$5x10^{-8}$ %	- / + / +	23%	$5x10^{-8}$ %	+/ + / +	25%	$5x10^{-8}$ %	+ / + / +
21%	$5x10^{-6}$ %	-/ + / +	23%	$5x10^{-6}$ %	+ / + / +	25%	$5x10^{-6}$ %	+ / + / +
21%	$5x10^{-5}$ %	+ / + / +	23%	$5x10^{-5}$ %	+ / + / +	25%	$5x10^{-5}$ %	+ / + / +
21%	$5x10^{-4}$ %	+ / + / +	23%	$5x10^{-4}$ %	+ / + / +	25%	$5x10^{-4}$ %	+ / + / +
21%	$5x10^{-3}$ %	+ / + / +	23%	$5x10^{-3}$ %	+ / + / +	25%	$5x10^{-3}$ %	+ / + / +

Table 18a. Experiment with tryptone and glucose solved in ethylacetat (mix for 1-2 hours at 50°C) as stock solution (50 mg tryptone and 10 mg glucose in 10 ml triflourethanol).

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm^3	PS I 10^9 cfu/mm^3	Enzyme Mix* 20 mg/mm^3
Bacterial Cocktail A 10^6 cfu/mm^3	PS II 10^9 cfu/mm^3	Enzyme Mix* 10 mg/mm^3
Homogenate II 10^8 cfu/mm^3	HII-1 10^9 cfu/mm^3	Ringer Solution
Homogenate II 10^6 cfu/mm^3	HII-2 10^9 cfu/mm^3	

Table 18b. Pipetting scheme. * Equal amount of Proteinase K, Chymotrypsine and Trypsin.

After 16h at 4°C

After 16h at room teperatur

After 16h at 37 °C

Column 1	Column 2
HII-2 10^9 cfu/mm^3	Ringer Solution
HII-2 (1:1000 dilution) ~10^6 cfu/mm^3	PS II 10^9 cfu/mm^3

Table 18c. Pipetting scheme. Ringer Solution as negative control.

After 4h at 4 °C

After 4h at r. t.

4.2 Bacterial Degradation of Cooking Oil as Carbon Source

It is generally known that bacteria, especially *Pseudomonas*, are able to transform vegetable (sunflower) oils and animal (pork and beef) fat, followed with excretion of lipases.

PLGA concentration	Desmodur (triisocyanat)	Sensor sensitivity 4°C / RT / 37°C
21%	$5x10^{-8}$ %	- / + / +
22%	$5x10^{-8}$ %	- / + / +
22%	$5x10^{-6}$ %	- / + / +
22%	$5x10^{-3}$ %	- / + / +
23%	$5x10^{-8}$ %	+ / + / +
23%	$5x10^{-6}$ %	- / + / +
25%	$5x10^{-3}$ %	+ / + / +
25%	$5x10^{-3}$ %	+ / + / +

Table 19a. Experiment with sunflower oil (1-20 µl/2ml), dissolved in ethylacetate.

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	Homogenate II 10^8 cfu/mm³	Enzyme Mix* 20 mg/mm³
Bacterial Cocktail A 10^6 cfu/mm³	Homogenate II 10^6 cfu/mm³	Enzyme Mix* 10 mg/mm³
Bacterial Cocktail C 10^6 cfu/mm³		Ringer Solution

Table 19b. Pipetting scheme. * Equal amount of Proteinase K, Chymotrypsine and Trypsine.

After 4h at 37 °C

After 16h at 37 °C

After 16h at r.t.

After 16h at 37 °C

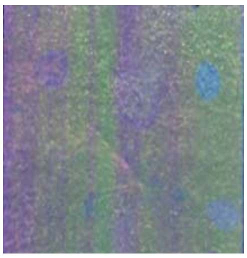
4.3 Sunflower Oil and Rising Desmodur (Triisocyanat) Concentration

PLGA concentration	Desmodur (triisocyanat)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (triisocyanat)	Sensor sensitivity 4°C / RT / 37°C
22%	$5x10^{-3}$ %	- / - / -	22%	1,6 %	- / - / +/-
22%	$5x10^{-2}$ %	- / - / -	22%	1,8 %	- / - / +/-
22%	$5x10^{-1}$ %	- / - / -	22%	2,2%	- / - / +/-
22%	1%	- / - / +/-	22%	2,4%	- / +/- / +/-
22%	1,2%	- / - / +/-	22%	2,6%	- / - / +/-
22%	1,4%	- / - / +/-	22%	3%	- / - / +/-

Table 20a. Experiment with sunflower oil and increased desmodur (triisocyanat) concentration ($5x10^{-3}$ % - 3%), performed in ethylacetat as a solvent.

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	Homogenate II 10^8 cfu/mm³	Enzyme Mix* 20 mg/mm³
Bacterial Cocktail A 10^6 cfu/mm³	Homogenate II 10^6 cfu/mm³	Enzyme Mix* 10 mg/mm³
Bacterial Cocktail C 10^6 cfu/mm³		Ringer Solution

Table 20b. Pipetting scheme. * Equal amount of Proteinase K, Chymotrypsine and Trypsine.


After 16h at 37 °C

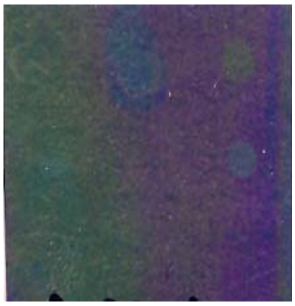

After 16h at 4°C

4.4 PLGA and Vegetable Butter Fat

PLGA concentration	Desmodur (triisocyanat)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (triisocyanat)	Sensor sensitivity 4°C / RT / 37°C
21%	$5x10^{-8}$ %	+/- / +/- / +	23%	$5x10^{-8}$ %	- / +/- / +/-
21%	$5x10^{-6}$ %	+/- / +/- / +	23%	$5x10^{-7}$ %	- / +/- / -
21%	$5x10^{-5}$ %	- / +/- / +	23%	$5x10^{-6}$ %	- / + / +
21%	$5x10^{-4}$ %	- / +/- / +/-	23%	$5x10^{-5}$ %	- / +/- / -
21%	$5x10^{-3}$ %	- / +/- / +/-	23%	$5x10^{-4}$ %	- / +/- / +/-
21%	$5x10^{-2}$ %	- / +/- / +/-	23%	0,7%	- / +/- / +/-
21%	$5x10^{-1}$ %	- / +/- / +/-	23%	0,8%	- / +/- / +/-
21%	1 %	- / +/- / +/-	23%	1 %	- / +/- / -

Table 21a. Experiment with vegetable fat (from sunflower, canola and corn) dissolved in ethylacetat at room temperature (55 mg butter/10 ml ethylacetat).

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	Homogenate II 10^8 cfu/mm³	Enzyme Mix* 20 mg/mm³
Bacterial Cocktail A 10^6 cfu/mm³	Homogenate II 10^6 cfu/mm³	Enzyme Mix* 10 mg/mm³
Bacterial Cocktail C 10^6 cfu/mm³		Ringer Solution



After 4h at 37 °C

Table 21b. Pipetting scheme. * Equal amount of Proteinase K, Chymotrypsine and Trypsine.

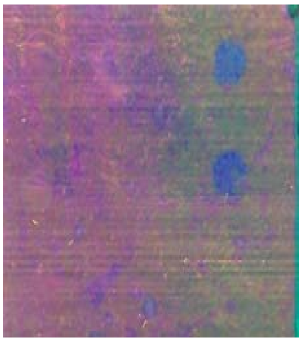
4.5 Glyceryl-Tributyrate and Increasing Amount of Desmodur (triisocyanate)

Tributyrate is an isomeric glyceril ester of butyric acid and is a main component of butter. It is commonly used for identification of microorganisms which can produce lipolytic enzymes. Furthermore, glycerine supports bacterial growth and is mostly added to bacteria when they have to be stored for a longer period at low temperatures. Mixing these two solutions was a great combination for the increasing of sensor sensitivity.

PLGA concentration	Desmodur (triisocyanat)	Sensor sensitivity 4°C / RT / 37°C
20%	$5x10^{-5}$ %	- / - / -
20%	$5x10^{-3}$ %	- / - / +
20%	$5x10^{-1}$ %	- / + / +
21%	$5x10^{-5}$ %	- / - / +/-
21%	$5x10^{-3}$ %	- / +/- / +/-
21%	$5x10^{-1}$ %	+/- / + / +
22%	$5x10^{-5}$ %	- / - / -
22%	$5x10^{-3}$ %	- / - / +/-
22%	$5x10^{-1}$ %	- / + / +

Table 22a. Experiment with glyceryl-tributyrate and increasing concentration of desmodur (triisocyanat), using ethylacetat as a solvent.

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	Homogenate II 10^8 cfu/mm³	Enzyme Mix* 20 mg/mm³
Bacterial Cocktail A 10^6 cfu/mm³	Homogenate II 10^6 cfu/mm³	Enzyme Mix* 10 mg/mm³
Bacterial Cocktail C 10^6 cfu/mm³		Ringer Solution



After 4h at room temperatur

Table 22b. Pipetting scheme. * Equal amount of Proteinase K, Chymotrypsine and Trypsine.

4.6. Tributyrate and Rising Concentration of Desmodur (diisocyanate)

PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C
20%	$5x10^{-5}$ %	- / - / +/-
20%	$5x10^{-3}$ %	- / + / +
20%	$5x10^{-1}$ %	+ / + / +
21%	$5x10^{-5}$ %	+ / + / +
21%	$5x10^{-3}$ %	+ / + / +
21%	$5x10^{-1}$ %	+ / + / +
22%	$5x10^{-5}$ %	+ / + / +
22%	$5x10^{-3}$ %	+ / + / +
22%	$5x10^{-1}$ %	+ / + / +

Table 23a. Experiment with glyceryl-tributyrate and rising concentration of desmodur (diisocyanate), using ethylacetate as a solvent.

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	Homogenate II 10^6 cfu/mm³	Enzyme Mix* 20 mg/mm³
Bacterial Cocktail A 10^6 cfu/mm³	HII-2 10^6 cfu/mm³	Enzyme Mix* 10 mg/mm³
Homogenate II 10^8 cfu/mm³	HII-2 1:100 dilution	Ringer Solution

After 16h at room temperatur

Table 23b. Pipetting scheme. * Equal amount of Proteinase K, Chymotrypsin and Trypsin.

PLGA concentration	Desmodur (diisocyanat)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (diisocyanat)	Sensor sensitivity 4°C / RT / 37°C
20%	$5x10^{-8}$ %	- / +/- / -	21%	$5x10^{-8}$ %	+/- / + / +
20%	$5x10^{-6}$ %	- / + / +	21%	$5x10^{-6}$ %	+/- / + / +
20%	$5x10^{-3}$ %	+/- / +/- / +/-	21%	$5x10^{-3}$ %	- / + / +
20%	$5x10^{-1}$ %	+/- / +/- / +/-	21%	$5x10^{-1}$ %	+ / + / +
20%	1%	+ / + / +	21%	1%	+ / + / +
PLGA concentration	**Desmodur (diisocyanat)**	**Sensor sensitivity 4°C / RT / 37°C**	**PLGA concentration**	**Desmodur (diisocyanat)**	**Sensor sensitivity 4°C / RT / 37°C**
23%	$5x10^{-8}$ %	- / + / +	24%	$5x10^{-8}$ %	- / + / +
23%	$5x10^{-6}$ %	- / + / +	24%	$5x10^{-6}$ %	+/- / + / +
23%	$5x10^{-3}$ %	- / + / +	24%	$5x10^{-3}$ %	- / + / +
23%	$5x10^{-1}$ %	- / + / +	24%	$5x10^{-1}$ %	+/- / + / +
23%	1%	+ / + / +	24%	1%	+/- / + / +

Table 24a. Experiments with glyceryl-tributyrate stock solution (1ml glycerine-tributyrin in 10 ml ethylacetate as a solvent).

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm^3	HII-2 10^9 cfu/mm^3	Bacterial Cocktail D 10^8 cfu/mm^3
Bacterial Cocktail A 10^6 cfu/mm^3	HII-A 10^7 cfu/mm^3	Bacterial Cocktail D 10^6 cfu/mm^3
Homogenate II 10^8 cfu/mm^3	HII-B 10^9 cfu/mm^3	Phospholipase
Homogenate II 10^6 cfu/mm^3	HII-B 1:1000 dilution	Ringer Solution

After 16h at 37 °C

Table 24b. Pipetting scheme. * Equal amount of

Proteinase K, Chymotrypsine and Trypsine.

4.7 PLGA with Glyceryl-Tributyrate and Sunflower Oil without Desmodur

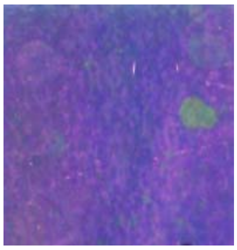
PLGA concentration	Sensor sensitivity 4°C / RT / 37°C
20%	- / + / +
20%	+/- / + / +
20%	- / + / +
21%	+/- / + / +
21%	+/- / + / +
21%	+/- / + / +
22%	+/- / + / +
22%	+/ + / +
22%	+/ + / +

Table 25a. Experiment with glyceryl-tributyrate and vegetable oil, but without desmodur binder. Ethylacetate was used as a solvent.

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	Homogenate II 10^6 cfu/mm³	HII-B 10^8 cfu/mm³
Bacterial Cocktail A 10^6 cfu/mm³	HII-2 10^6 cfu/mm³	Enzyme Mix* 20 mg/mm³
Homogenate II 10^8 cfu/mm³	HII-A 10^7 cfu/mm³	Ringer Solution

Table 25b. Pipetting scheme. * Equal amount of Proteinase K, Chymotrypsine and Trypsine.

After 6h at r.t.



After 6h at r.t.

4.8 Trypton and Cooking Oil

PLGA concentration	Desmodur (diisocyanat)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (diisocyanat)	Sensor sensitivity 4°C / RT / 37°C
20%	$5x10^{-8}$ %	+/-/ +/- / +	21%	$5x10^{-8}$ %	+/-/ +/- / +/-
20%	$5x10^{-6}$ %	-/ + / +	21%	$5x10^{-6}$ %	+/-/ +/- / +
20%	$5x10^{-3}$ %	+/-/ + / +	21%	$5x10^{-3}$ %	+/ +/ +
20%	$5x10^{-2}$ %	+/-/ + / +	21%	$5x10^{-2}$ %	+/ + / +
20%	1%	+/-/ + / +	21%	1%	+/ + / +
PLGA concentration	**Desmodur (diisocyanat)**	**Sensor sensitivity 4°C / RT / 37°C**	**PLGA concentration**	**Desmodur (diisocyanat)**	**Sensor sensitivity 4°C / RT / 37°C**
23%	$5x10^{-8}$ %	+/-/ + / +	24%	$5x10^{-8}$ %	-/ + / +
23%	$5x10^{-6}$ %	+/-/ + / +	24%	$5x10^{-6}$ %	+/ + / +
23%	$5x10^{-3}$ %	+/-/ + / +/-	24%	$5x10^{-3}$ %	+/ + / +
23%	$5x10^{-2}$ %	+/ + / +	24%	$5x10^{-2}$ %	+/ + / +
23%	1%	+/ + / +	24%	1%	+/ + / +
PLGA concentration	**Desmodur (diisocyanat)**	**Sensor sensitivity 4°C / RT / 37°C**	**PLGA concentration**	**Desmodur (diisocyanat)**	**Sensor sensitivity 4°C / RT / 37°C**
25%	$5x10^{-8}$ %	-/ + / +	27%	$5x10^{-8}$ %	+/ + / +
25%	$5x10^{-6}$ %	-/ + / +	27%	$5x10^{-6}$ %	+/ + / +
25%	$5x10^{-3}$ %	+/-/ + / +	27%	$5x10^{-3}$ %	+/ + / +
25%	$5x10^{-2}$ %	+/-/ + / +	27%	$5x10^{-2}$ %	+/ + / +
25%	1%	+/-/ + / +	23%	1%	+/ + / +

Table 26a. Experiment with trypton and cooking oil (sunflower) in a day before prepared stock of 50 mg tryptone in 10 ml ethylacetat, disolved at 50°C (~2 hours).

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	HII-2 10^9 cfu/mm³	Bacterial Cocktail D 10^8 cfu/mm³
Bacterial Cocktail A 10^6 cfu/mm³	HII-A 10^7 cfu/mm³	Bacterial Cocktail D 10^6 cfu/mm³
Homogenate II 10^8 cfu/mm³	HII-B 10^9 cfu/mm³	Phospholipase
Homogenate II 10^6 cfu/mm³	HII-B 1:1000 dilution	Ringer Solution

After 16h at 37 °C

After 16h at 37 °C

After 16h at room temperatur

Table 26b. Pipetting scheme. ***** Equal amount of Proteinase K, Chymotrypsine and Trypsine.

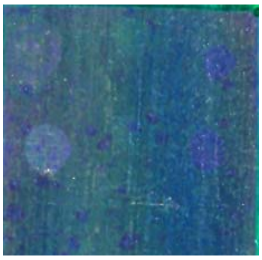
4.9 PLGA and Tri-Natrium-Citrate

PLGA concentration	Desmodur (diisocyanat)	Sensor sensitivity 4°C / RT / 37°C
26%	$5x10^{-8}$ %	-/ + / +
26%	$5x10^{-6}$ %	-/ + / +
26%	$5x10^{-3}$ %	-/ + / +
26%	1,2 %	-/ + / +

Table 27a. Experiment with tri-natrium-citrate x $2H_2O$ as a substrate, solved in ddH_2O (22,8 mg tri-natrium-citrate x $2H_2O$/55 µl ddH_2O).

Column 1	Column 2	Column 3
Homogenate II 10^8 cfu/mm³	GSP 10^6 cfu/mm³	Enzyme Mix* 20 mg/mm³
Homogenate II 10^6 cfu/mm³	Ringer Solution	Enzyme Mix* 10 mg/mm³

Table 27b. Pipetting scheme. ***** Equal amount of Proteinase K, Chymotrypsine and Trypsine.



After 16h at 37 °C

After 4h at room temperatur

After 16h at room temperatur

4.10 Lactose as the Substrate for the PLGA-Layer

PLGA concentration	Desmodur (diisocyanat)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (diisocyanat)	Sensor sensitivity 4°C / RT / 37°C
21%	$5x10^{-8}$ %	-/ +/- / +/-	23%	$5x10^{-8}$ %	-/ +/ +
21%	$5x10^{-6}$ %	-/ + / +/-	23%	$5x10^{-6}$ %	-/ +/ +
21%	$5x10^{-3}$ %	-/ + / +/-	23%	$5x10^{-3}$ %	-/ +/ +
21%	$5x10^{-1}$ %	-/ + / +/-	23%	$5x10^{-1}$ %	-/ +/ +
21%	1%	-/ + / +/-	23%	1%	-/ + / +

Table 28a. Experiment with lactose (8,6 mg lactose in 10 ml ethyl acetate) and induction of microorganisms to excrete enzymes. Lactose is used as stimulus for lipolytic enzyme secretion especially for the *Lactobacillus* species.

Column 1	Column 2	Column 3
M1 10^4 cfu/mm³	G1 10^3 cfu/mm³	HII-B 10^6 cfu/mm
M1 10^3 cfu/mm³	G1 10^2 cfu/mm³	Bacterial Cocktail D 10^8 cfu/mm³
M2 10^4 cfu/mm³	HII-A 10^7 cfu/mm³	Bacterial Cocktail D 10^6 cfu/mm³
M2 10^3 cfu/mm³	HII-B 10^8 cfu/mm	Ringer Solution

Table 28b. Pipetting scheme. * Equal amount of

Proteinase K, Chymotrypsine and Trypsine.

After 4h at r.t.

After 16h at r.t.

After 16h at 37°C

4.11 Glucose and Sunflower Oil

PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C
21%	$5x10^{-8}$ %	+/ +/ +/-	23%	$5x10^{-8}$ %	-/ +/ +
21%	$5x10^{-6}$ %	+/ +/ +/-	23%	$5x10^{-6}$ %	-/ +/ +
21%	$5x10^{-3}$ %	+/ + / +	23%	$5x10^{-3}$ %	+/ + / +
21%	$5x10^{-1}$ %	+/ + / +	23%	$5x10^{-1}$ %	+/ + / +
21%	1%	+/ + / +	23%	1%	+/ + / +

Table 29a. Stock of glucose and sunflower oil were prepared in ethyl acetate (7 mg glucose in 10 ml solvent). PLGA sensors whith glucose and oil as a substrate showed the best signal even at 4°C, after only 4 hours.

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	HII-2 10^9 cfu/mm³	Bacterial Cocktail D 10^8 cfu/mm³
Bacterial Cocktail A 10^6 cfu/mm³	HII-A 10^7 cfu/mm³	Bacterial Cocktail D 10^6 cfu/mm³
Homogenate II 10^8 cfu/mm³	HII-B 10^9 cfu/mm³	Phospholipase
Homogenate II 10^6 cfu/mm³	HII-B 1:1000 dilution	Ringer Solution

After 4h at 37 °C

Table 29b. Pipetting scheme. * Equal amount of

Proteinase K, Chymotrypsine and Trypsine.

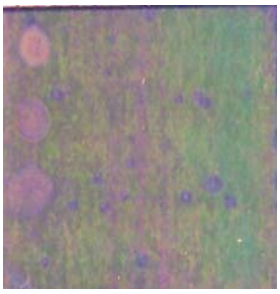
4.12 PLGA and Tween 20

PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C
21%	$5x10^{-8}$ %	-/ -/ +	23%	$5x10^{-8}$ %	-/ +/ +
21%	$5x10^{-6}$ %	+/ +/-/ +	23%	$5x10^{-6}$ %	+/ +/ +
21%	$5x10^{-3}$ %	-/ -/ +	23%	$5x10^{-3}$ %	+/ + / +
21%	$5x10^{-1}$ %	+/ +/-/ +	23%	$5x10^{-1}$ %	-/ + / +
21%	1%	+/ +/-/ +	23%	1%	-/ +/- / +

Table 30a. Tween 20 stock solution was prepared in ethyl acetate in 1:100 dilutions.

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	HII-2 10^9 cfu/mm³	Bacterial Cocktail D 10^8 cfu/mm³
Bacterial Cocktail A 10^6 cfu/mm³	HII-A 10^7 cfu/mm³	Bacterial Cocktail D 10^6 cfu/mm³
Homogenate II 10^8 cfu/mm³	HII-B 10^9 cfu/mm³	Phospholipase
Homogenate II 10^6 cfu/mm³	HII-B 1:1000 dilution	Ringer Solution

Table 30b. Pipetting scheme. * Equal amount of Proteinase K, Chymotrypsine and Trypsine



After 6h at 37 °C

After 16h at 37 °C

4.13 Glucose, Lactose and Sunflower Oil as Energy Sources

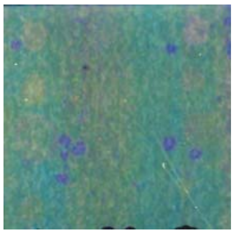
PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C
21%	$5x10^{-8}$ %	+/-/ +/ +/-	23%	$5x10^{-8}$ %	+/ +/ +
21%	$5x10^{-6}$ %	+/ +/ +	23%	$5x10^{-6}$ %	+/ +/ +
21%	$5x10^{-3}$ %	-/ +/ +	23%	$5x10^{-3}$ %	+/ + / +
21%	$5x10^{-1}$ %	+/ +/ +	23%	$5x10^{-1}$ %	+/ + / +
21%	1%	+/ +/ +	23%	1%	+/ + / +

Table 31a. Substrate combination of glucose, lactose and sunflower oil showed excellent results at 4°C after 6 hours.

Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm^3	HII-2 10^9 cfu/mm^3	Bacterial Cocktail D 10^8 cfu/mm^3
Bacterial Cocktail A 10^6 cfu/mm^3	HII-A 10^7 cfu/mm^3	Bacterial Cocktail D 10^6 cfu/mm^3
Homogenate II 10^8 cfu/mm^3	HII-B 10^9 cfu/mm^3	Phospholipase
Homogenate II 10^6 cfu/mm^3	HII-B 1:1000 dilution	Ringer Solution

After 6h at 37°C

After 16h at 37 °C



After 16h at 37 °C

Table 31b. Pipetting scheme. * Equal amount of Proteinase K, Chymotrypsine and Trypsine.

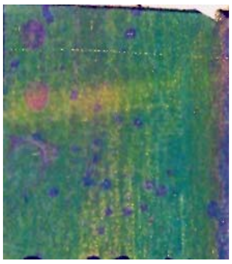
4.14 Glucose, Glyceryl-Tributyrate and Sunflower Oil

PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C
21%	5x10^{-8} %	-/ +/-/ +	23%	5x10^{-8} %	+/ +/ +
21%	5x10^{-6} %	-/ +/ +	23%	5x10^{-6} %	+/ +/ +
21%	5x10^{-3} %	-/ +/ +	23%	5x10^{-3} %	+/ + / +
21%	5x10^{-1} %	-/ +/ +	23%	5x10^{-1} %	-/ + / +
21%	1%	+/ +/ +	23%	1%	+/-/ + / +

Table 32a. Bacterial induction for the enzyme production using glucose, glyceryl-trybutyrate and sunflower oil in 1:1:1 ratio.

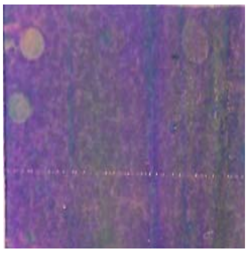
Column 1	Column 2	Column 3
Homogenate III 10^{6} cfu/mm^{3}	HII-2 10^{9} cfu/mm^{3}	Bacterial Cocktail D 10^{8} cfu/mm^{3}
Homogenate IV 10^{7} cfu/mm^{3}	HII-A 10^{7} cfu/mm^{3}	Bacterial Cocktail D 10^{6} cfu/mm^{3}
Homogenate II 10^{8} cfu/mm^{3}	HII-B 10^{9} cfu/mm^{3}	Phospholipase
Homogenate II 10^{6} cfu/mm^{3}	HII-B 1:1000 dilution	Ringer Solution

Table 32b. Pipetting scheme.



After 6h at 37°C

After 6h at 37°C



After 16h at room temperatur

4.15 PLGA and Natrium-dihydrogen-phosphate

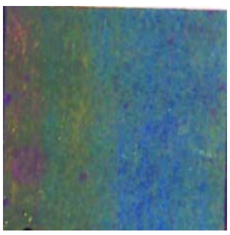
PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C
21%	$5x10^{-8}$ %	-/ +/ +	23%	$5x10^{-8}$ %	+/ +/ +
21%	$5x10^{-6}$ %	+/ +/ +	23%	$5x10^{-6}$ %	+/ +/ +
21%	$5x10^{-3}$ %	+/ +/ +	23%	$5x10^{-3}$ %	+/ +/ +
21%	$5x10^{-1}$ %	+/ +/ +	23%	$5x10^{-1}$ %	+/ +/ +
21%	1%	+/ +/ +	23%	1%	+/ +/ +

Table 33a. NaH_2PO_4 as a substrate for the bacterial induction and enzyme excretion.

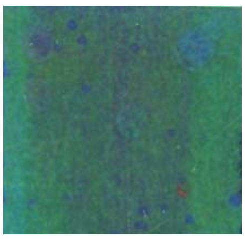
Column 1	Column 2	Column 3
Homogenate III 10^6 cfu/mm³	HII-2 10^9 cfu/mm³	Bacterial Cocktail D 10^8 cfu/mm³
Homogenate IV 10^7 cfu/mm³	HII-A 10^7 cfu/mm³	Bacterial Cocktail D 10^6 cfu/mm³
Homogenate II 10^8 cfu/mm³	HII-B 10^9 cfu/mm³	Phospholipase
Homogenate II 10^6 cfu/mm³	HII-B 1:1000 dilution	Ringer Solution

Table 33b. Pipetting scheme.

After 6h at 37°C



After 6h at 4°C



After 16h at r.t.

4.16 Powdered Milk as Substrate

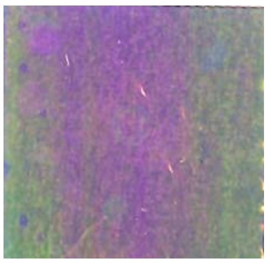
PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration	Desmodur (diisocyanate)	Sensor sensitivity 4°C / RT / 37°C
21%	$5x10^{-8}$ %	-/ +/-/ +	23%	$5x10^{-8}$ %	+/ +/ +
21%	$5x10^{-6}$ %	+/-/ +/-/ +	23%	$5x10^{-6}$ %	+/ +/ +
21%	$5x10^{-3}$ %	+/-/ +/ +	23%	$5x10^{-3}$ %	-/ +/ +
21%	$5x10^{-1}$ %	+/-/ +/ +	23%	$5x10^{-1}$ %	+/ +/ +
21%	1%	+/ +/ +	23%	1%	+/ +/ +

Table 34a. Milk powder as a substrate for the bacterial induction and exogenous lipase production.

Column 1	Column 2	Column 3
Homogenate III 10^6 cfu/mm³	HII-2 10^9 cfu/mm³	Bacterial Cocktail D 10^8 cfu/mm³
Homogenate IV 10^7 cfu/mm³	HII-A 10^7 cfu/mm³	Bacterial Cocktail D 10^6 cfu/mm³
Homogenate II 10^8 cfu/mm³	HII-B 10^9 cfu/mm³	Phospholipase
Homogenate II 10^6 cfu/mm³	HII-B 1:1000 dilution	Ringer Solution

Table 34b. Pipetting scheme.

After 6h at 37 °C



After 16h at room temperatur

5. Fabry-Perot Setup with PLGA as Interlayer

Fabry-Perot sensor setup according to [18] and using PLGA [poly(lactic-coglycolic acid)], as distance layer did not show the expected results. It was possible to sputter the gold nanoparticles which were floated on PLGA interlayer. However, this kind of sensor setup did not show necessary stability and both layers, PLGA distance layer and gold nanoparticles on top of it, were dissolved after sensor testing with bacteria and after a washing procedure with demineralized water.

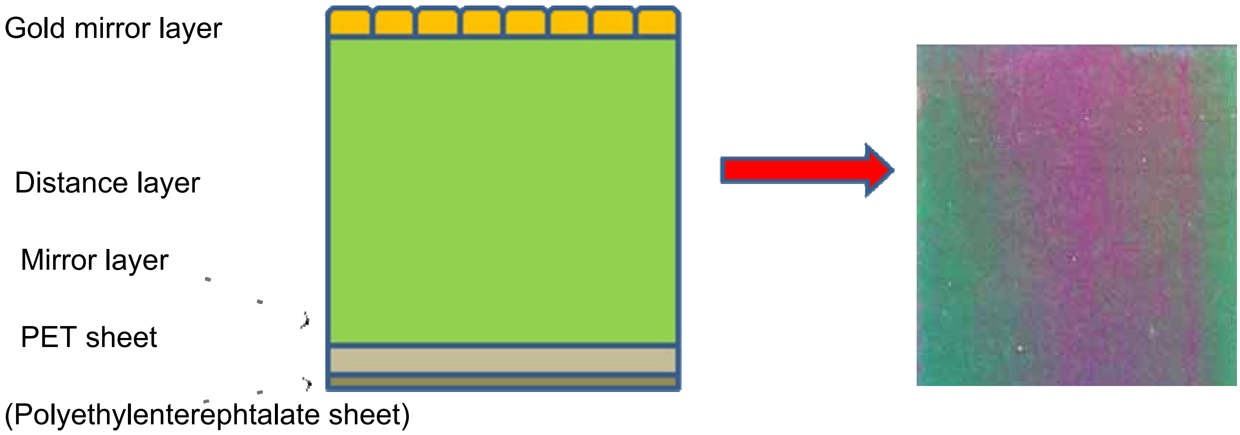
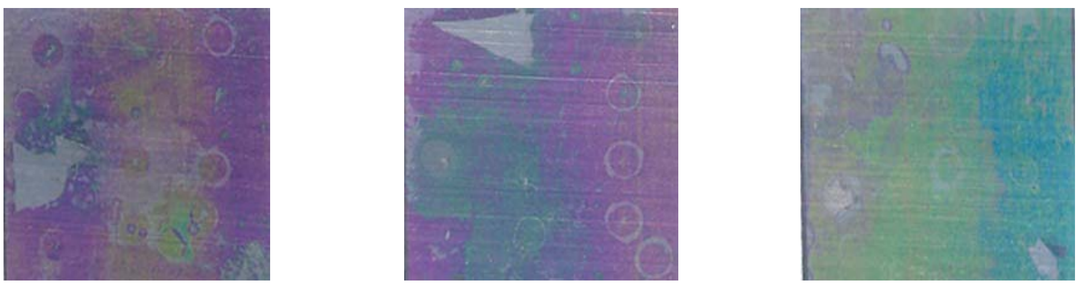


Figure 11. Fabry-Perot Sensor Setup according to [18] (left) and sensor setup with PLGA as interlayer (right).



After 4h at 37 °C
Sputtering: 10 sec

After 4h at room temperatur
Sputtering: 10 sec

After 4h at 4°C
Sputtering: 10 sec

6. Liquid Media and Enzyme Excretion

Liquid media are commonly used for inhibition or promotion of microbial growth. They can induce certain metabolic pathways or provoke specific reactions. The liquid media in this work were used to stimulate bacteria for production of lipolytic enzymes. Following the bacterial growth in liquid media and mesurement of enzyme activity at different points of bacterial logaritmic phase, it was possible to find correlation between total bacterial count from logarithmic phase and lipolytic enzymes (aminopeptidase) which were secreted during that time. It was shown that meat bacteria (*Pseudomonas* and *Enterobacter*) at the begining of logarithmic phase in bacterial count of 10^7 cfu/mm^3 produce the highest level of aminopeptidase.

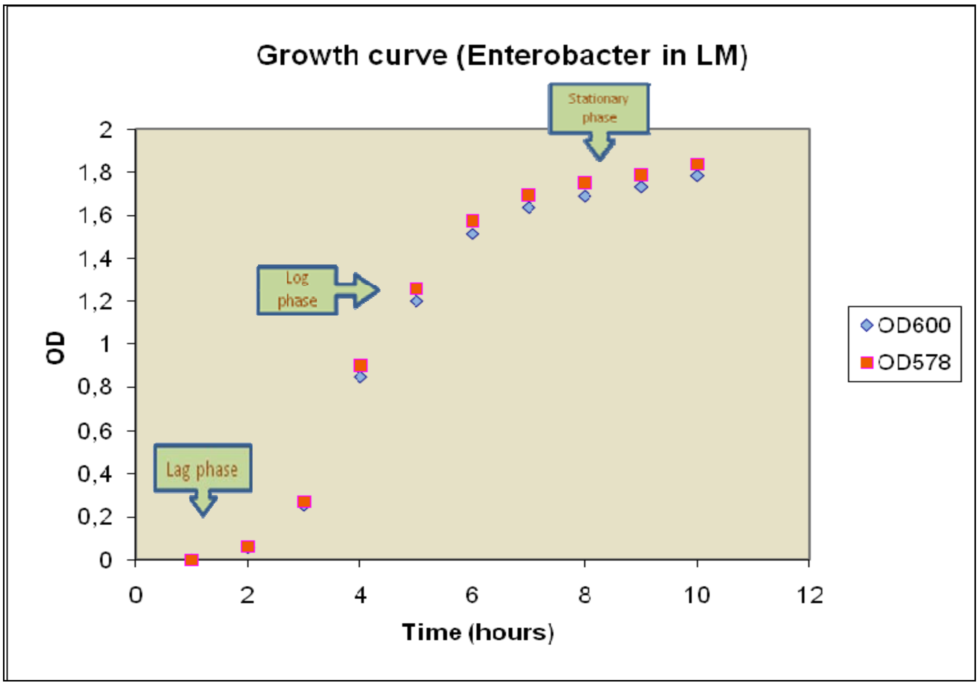


Diagram 3. Growing curve of Enterobacter in liquid media at 37 °C.

Enterobacter in Liquid Media			
Incubation time	**OD600**	**OD578**	**CFU/mL**
0	0	0	
2h	0,057	0,062	
3h	0,255	0,271	**$4*10^{5}$**
4h	0,847	0,899	**$4,9*10^{7}$**
4,5h	1,2	1,257	**$1,6*10^{7}$**
5h	1,513	1,572	**$2*10^{7}$**
5,5h	1,636	1,692	**$1,6*10^{8}$**
6h	1,689	1,75	
6,5 h	1,732	1,786	**$1,5*10^{9}$**
7h	1,784	1,835	

Table 35. Total bacterial count of the bacterial samples from log- and stationary-phase.

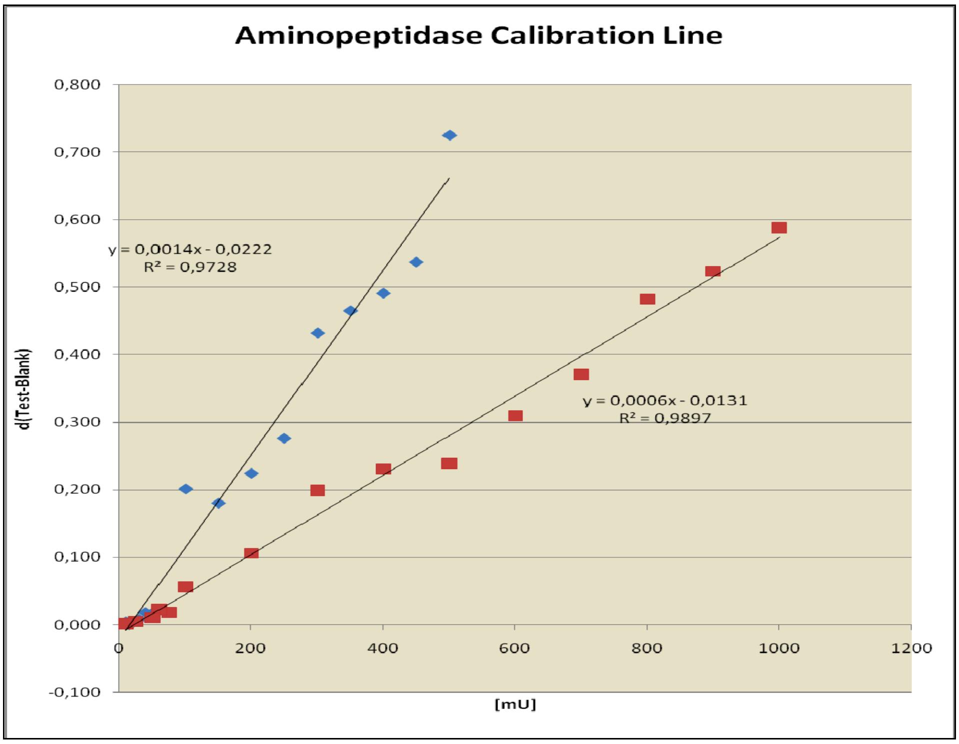


Diagram 4. Leucine-aminopeptidase calibration line

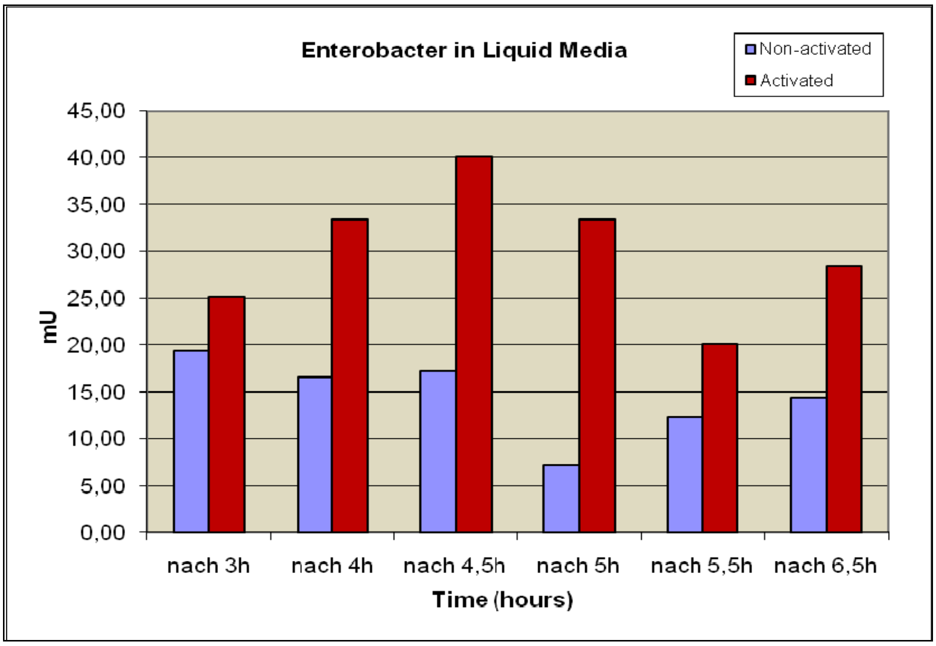


Diagram 5. Leucine-aminopeptidase activity of Enterobacter in liquid media.

Time (hours)	Non-activated (mU)	Activated (mU)	CFU/mL
3	19,43	25,17	4+E05
4	16,57	33,50	4,9+E07
4,5	17,29	40,17	1,6+E07
5	7,29	33,50	2+E07
6	12,29	20,17	1,6+E08
6,5	14,43	28,50	1,5+E09

Table 36. Correlation between Enterobacter counts in log-phase and secreted enzyme amount .

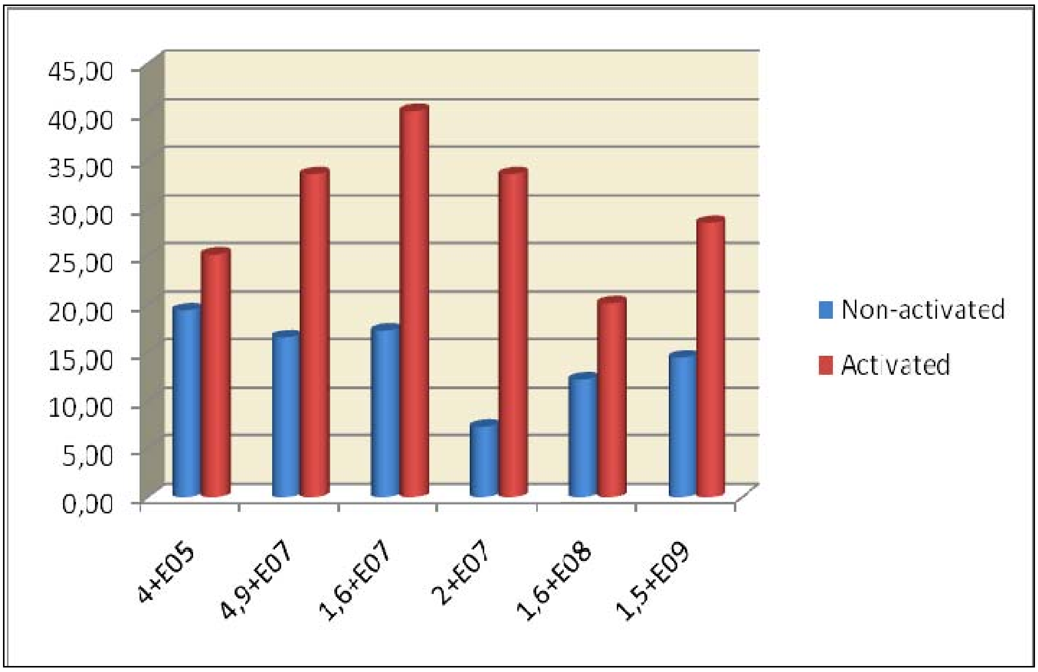


Diagram 6. Graphic presentation of correlation between total bacterial count of *Enterobacter* in liquid media and secreted leucine-aminopeptidase amount.

7. Screening of Lipolytic Activity

Bacteria isolated from spoiled meat (*Pseudomonas, Enterobacter, Proteus* and *Salmonella*) were screened for exogenous lipolytic activity, using tributyrin agar base and glycerin-tributyrate. Optimal cultural parameters influencing bacterial growth and production of lipolytic enzymes were also investigated. These four bacteria showed the maximal lipase yielded in bacterial count of 10^6 cfu/mm^3 and 10^8 cfu/mm^3, after incubation at 37°C after 24 hours. First evidence for lipolytic activity of meat bacteria incubated at 4°C was observed after three days. When the medium was modified and prepared using glucose monohydrate as carbon source, better lipase yields were gained. Incubation of the bacteria at 30°C also showed first evidence for lipolytic activity after 24 hours.

1 2 3 4

10^8-cfu/mm^3

10^6cfu/mm^3

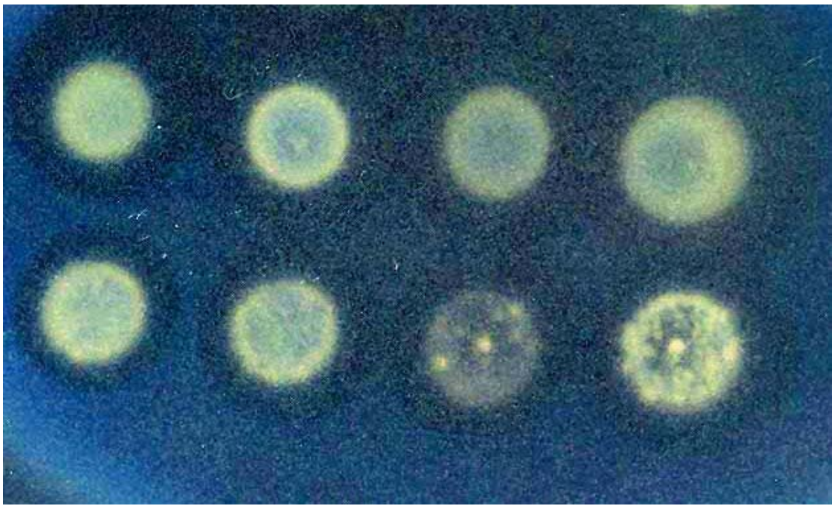


Figure 12. Lipolytic activity screen for Pseudomonas (1), Sallmonella (2), Enterobacter (3) and Proteus (4), using tributyrin basis agar. Incubation at 37 °C for 24 hours.

1 2 3 4

10^8-cfu/mm^3

10^6cfu/mm^3

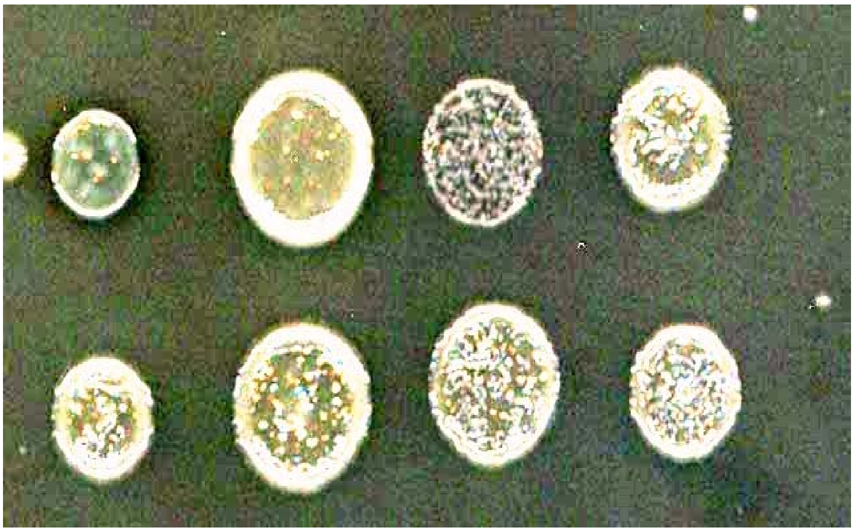


Figure 13. Lipolytic activity screening for Pseudomonas (1), Salmonella (2), Enterobacter (3) and Proteus (4), using tributyrin basis agar. Incubation: at 4 °C for three days.

8. Poly Lactic Acid (PLA) and Bacterial Induction

First idea for substrate induction of bacteria for better excretion of lipolytic enzymes was realized using Fabry-Perot sensor setup [18] with poly lactic acid as distance layer. By food spoilage and increased bacterial results in decrease or increase in pH-value of infected food sample. Bacteria such as *Pseudomonas, Enterobacter, Salmonella* or *Proteus* for example are able to utilize citrate as energy and carbon source and ammonium salt which alkalinizes the medium. The use of an appropriate pH-indicator (bromothymolblue in this case) causes color change. The same color reaction could appear on the sensor surface after bacterial degradation. Different microbial methabolic reactions in correlation with pH-value and pH-indicators were used to achieve the same efect in aboved sensor setup.

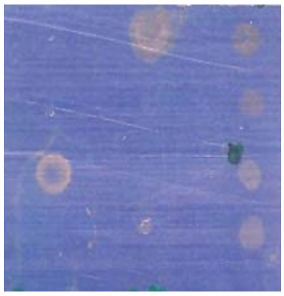
8.1 Tri-Natrium-Citrate and Bromothymol Blue

Poly lactic acid was used in 3,75% concentration, dissolved in tri-flouro-ethanol under addition of tri-natrium-citrate and bromothymol blue.

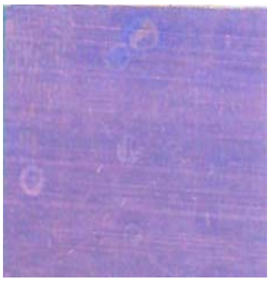
Column 1	Column 2	Column 3
Bacterial Cocktail A 10^8 cfu/mm³	Homogenate II 10^8 cfu/mm³	Enzyme Mix* 20 mg/mm³
Bacterial Cocktail A 10^6 cfu/mm³	Homogenate II 10^6 cfu/mm³	Enzym eMix* 15 mg/mm³
Homogenate I 10^8 cfu/mm³	Bacterial Cocktail C 10^7 cfu/mm³	Enzyme Mix* 10 mg/mm³
Homogenate I 10^6 cfu/mm³	Bacterial Cocktail C 10^6 cfu/mm³	Enzyme Mix* 5 mg/mm³
		Ringer Solution

Table 37. Pipetting scheme. ***** Equal amount of Proteinase K, Chymotrypsine and Trypsine.

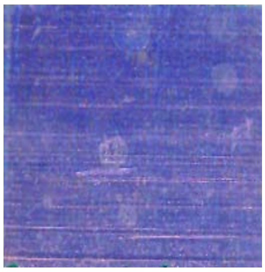
After 4h at room temperatur



After 4h at 37 °C



After 4h at 4 °C



After 16h at 4°C

Conclusion

An optical thin film sensor chip can detect bacterial decay of food through a specific color change. A biometic polymer which is integrated in such two layer sensor setup is degradable by lytic enzymes excreted by microorganisms in food decay. However, the polymer alone is not enough for induction of microorganisms to produce sufficient amount of lipolytic enzymes for its degradation. Investigating the charachteristics and biology of microorganisms and their metabolitic pathways, it was possible to see that

not all microorganisms produce the same concentration of lipolytic enzymes under the same conditions.

Simulating the spoilage conditions on the meat surface at the first place I used different kinds of media for bacterial growth and higher abbility for enzyme excretion. Furthermore, there was need for the polymer layer modification, too. Results of enzymatic assays (phospholipase c and aminopeptidase) showed that higher bacterial count does not mean immediately higher enzyme activity and better polymer degradation. My goal was, in correlation with bacterial counts from 10^6-10^8 cfu/mm^3 and lipolytic enzymes amount produced at that point, to optimize sensor sensitivity under addition of substrates which can be recognized and digested by investigated bacterial strains (*Pseudomonas, Enterobacter, Proteus, Salmonela, Serratia, Lactobacillus, Leuconostoc, Brochothrix and Acinetobacter*).

Followed with generally known bacterial features I tested their behaviour on the sensor surface under addition of glucose, lactose, trypton, tri-natrium-citrate, cooking oil, tween 20 and tributyrin to the polymer (PLGA) layer. With proper substrate amount and incubation conditions I was able to induce the above mentioned bacteria for better enzyme production reasulting stronger color change of degraded polymer (PLGA) layer . Under such conditions bacteria were not forced to grow faster, in contrary they continued the normal logarithmic growth, but only sensitivity of the sensor for secreted enzyme was higher.

Publication

Nadira Ibrišimović*, Margit Barth[1], Ulrich Bohrn[1], Mirza Ibrišimović[1], Fritz Pittner

Biomimetic sensor chip monitoring real-time food degradation: correlating chemical deterioration with microbiological status.

Department of Biochemistry and Cell Biology, University of Vienna and MFPL, Dr. Bohr Gasse 9, A-1030 Vienna, Austria

[1] contributed equally

* to whom all correspondence should be sent nadira.ibrisimovic@univie.ac.at

Keywords: biomimetic sensor chip, freshness sensor, meat decay monitoring, metal nanocluster sensor chip

Abstract

Conventional tests, currently in use for the detection and identification of food borne pathogens and also of microbial food deterioration are time consuming as they are based on conventional culturing techniques or monitoring environmental conditions like the increase of temperature or change in pH. These parameters do not reflect the real quality of the meat to be tested.

The aim of our approach was to create a simple and cheap sensor providing reasonable sensitivity and selectivity to indicate the bacterial infection in real time monitoring combined with a memory effect that cannot easily be corrupted.

Thus an optical thin film sensor chip was developed able to detect bacterial decay of food through a specific colour change. The design of the sensor relates to the phenomenon of “anomalous absorption”, which can best be described as a thin film enhanced absorption. A metal nanoparticle layer positioned at a well defined distance to a smooth metal surface shows that the minimum of spectral reflectivity strongly depends on the thickness of the distance layer. This setup represents a special kind of reflection interference filter. In such a sensor setup a biomimetic polymer is integrated which is degradable by lytic enzymes excreted by microorganisms in food decay. Meat deterioration under controlled conditions is correlated to the amount of enzymes secreted by microorganisms and the bacterial count. Thus, after incubation of the sensor setup with standard meat preparations, the enzymes released from decaying cell material change the thickness of the polymer layer and generate an easily visible colour change. This setup would be useful for integration into meat packaging.

Introduction:

Standard methods for the detection and identification of food degradation show high accuracy and low detection limits, but are generally expensive, time-consuming and require the use of highly trained personnel and laboratory equipment. The current tendency to carry out field monitoring has driven the development of biosensors as new analytical tools able to provide fast, reliable and sensitive measurements with lower cost. These biosensors for the moment do not compete with official analytical methods, but they can be used both – by regulatory authorities and by industry to provide enough information for routine testing and screening of samples [1].

Development and improvement of food processes is driven by the need for healthier, safer, more convenient, competitively superior and seasonally invariant foods and also more efficient processing plants with reduced waste. Online optical instruments such as refractometers, spectrophotometers, turbidity meters etc. that may be used to assess food composition have been developed. However, their applicability is often limited because of the complex composition of food interfering with the measurement and much stress has to be laid on correct sample preparation.

Available quick tests for food decay using parameters such as time, temperature or pH change are not really correlating to bacterial contamination. Monitoring of pH gives only indirect information and most tests are reversible, and therefore generally unapt to contribute to the safety of the meat supply chain. Microbiological tests concerning bacterial counts or strains are very time consuming as they rely on conventional culturing techniques and thus are far from real time monitoring. Especially when a consumer wants to get information on the quality of what he is buying in the shop, a quick real time test integrated into the package would be of high interest, as well as also the producer can show the hygienic level of his production line by such a device.

Meat decay is caused mainly by microorganisms excreting lytic enzymes. Both, rate of bacterial growth and enzymatic action are functions of time and temperature. Increasing temperature raises the amount of microorganisms and also the activity of the secreted enzymes, thus enhancing enzymatic deterioration of meat tremendously. (Fig. 1)

<Figure 1>

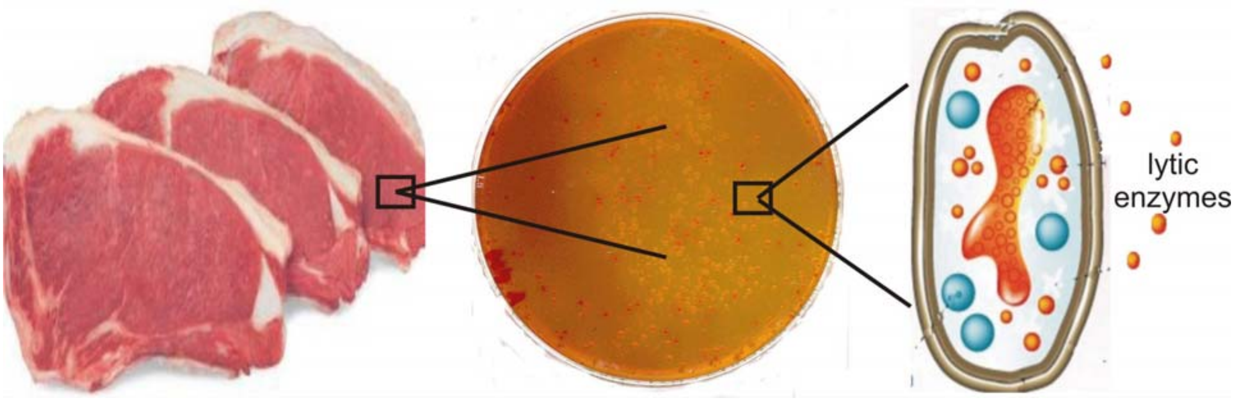


Fig. 1. Bacterial growth on meat surface causes excretion of lytic enzymes

In the last decades, the combination of knowledge in electrochemistry, biochemistry, physics and integrated circuit silicon technology made it possible to provide highly specific, sensitive, selective, accurate and reliable microsensors [2 – 6].

Due to the special optical behaviour of a metal island film and due to the thin film set up, this system shows characteristic spectral reflection behaviour, strongly dependent on the thickness of the transparent distance layer [7], in our case formed by the biomimetic polymer. The optical property of metal island films, necessary for our application, is the so called “Plasmon absorption”, a strong, broad-band absorption in the visible, due to the confinement of the conduction electron plasma in nanometric particles. This is in contrast to the unconfined electron movement in an extended metal, responsible for strong, unspecific reflectivity, well known as metallic glance. An absorbing thin film positioned at a defined distance to a metal mirror represents a special kind of reflection interference filter.

At an appropriate distance of the absorbing layer to the mirror, fields reflected by the mirror have the same phase at the position of the absorbing layer as the incident fields and, thus, by this feedback mechanism the effective absorption coefficient of the absorbing layer is strongly enhanced. This combination of the two phenomena plasmon absorption and optical interference is generally called “anomalous absorption”.

Fig. 2 shows the visual impression obtained by observation of the reflected light upon diffuse white-light illumination of the layer system used in our sensor set up in the interlayer optical thickness range 0 – 490 nm (optical thickness = geometrical thickness x effective refractive index) and some corresponding, measured reflection spectra.

<Figure 2>

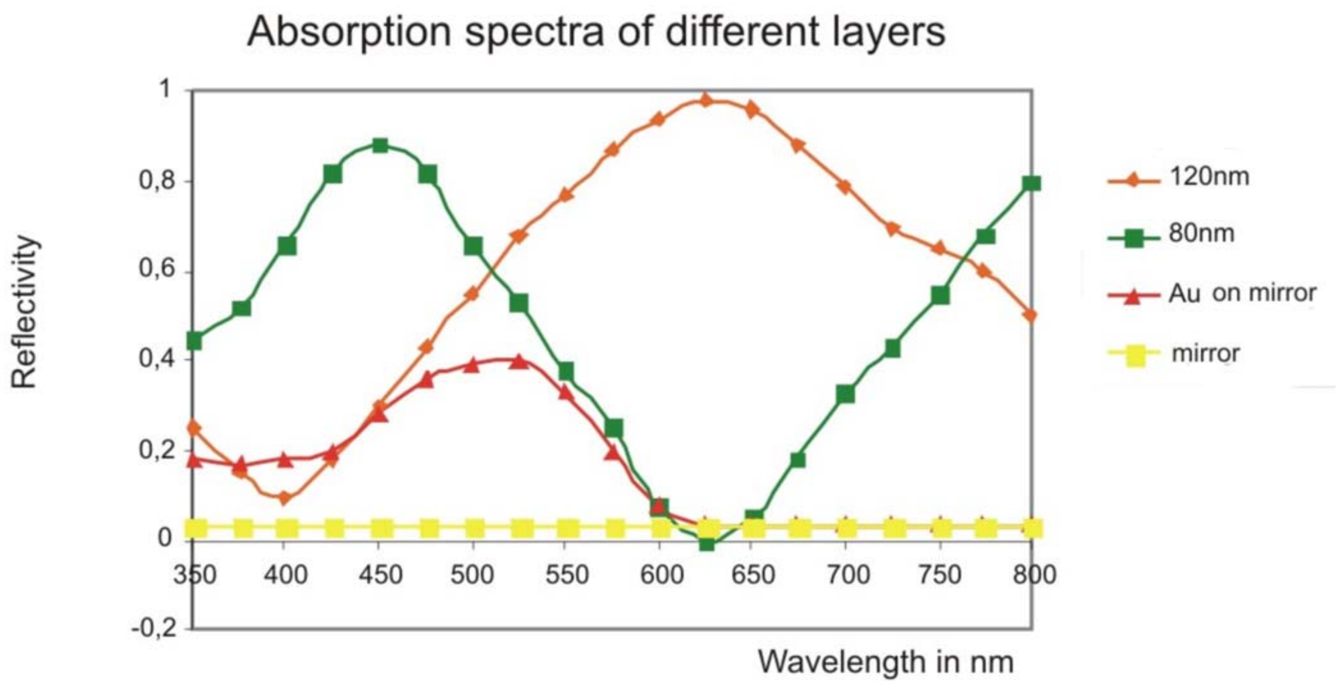


Fig. 2: Colour of the sensor as a function of thickness of the distance layer

Results and Discussion:

The setup of the sensor measuring bacterial decay of food is shown in Fig. 3 and assembled according to [8]. It has to be in permanent physical contact with meat or meat juice so that the lytic enzymes are able to diffuse through the highly permeable nanoparticle layer thus reaching the distance layer. It consists of a biomimetic polymer within a nanometric range and thus shows very fast response. The polymer chains are cleaved resulting in altered layer thickness and therefore a change in the colour can be observed by the naked eye. The thickness of the distance layer can be tuned by the viscosity and composition of the polymer solution and by the parameters of the printing process.

<Figure 3>

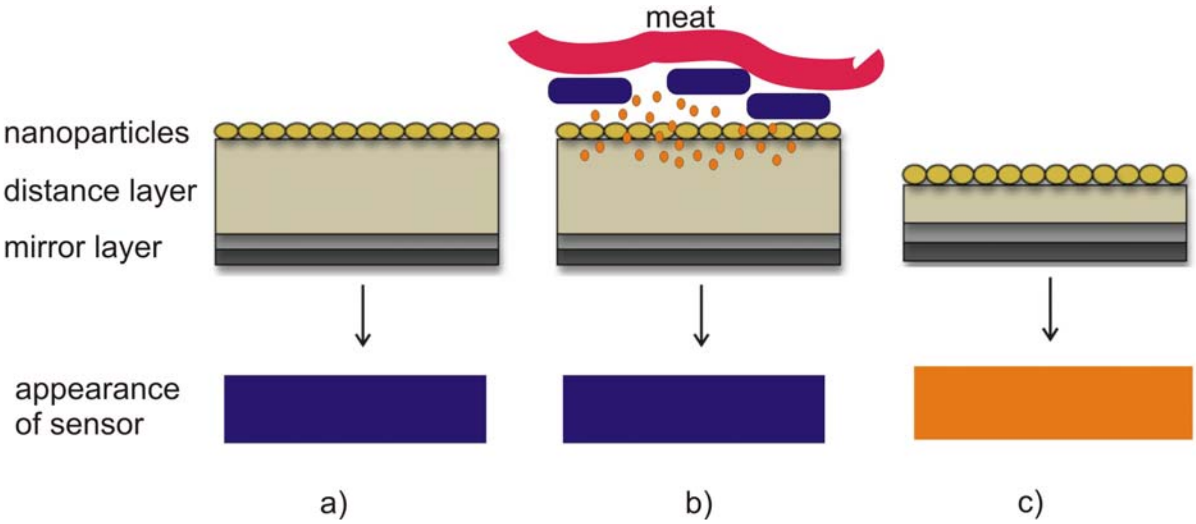


Fig. 3. Setup and function of the sensor: a) sensor setup b) sensor in contact with meat and bacteria c) sensor after degradation of the biomimetic polymer. The colours shown in the lower level are only examples and are dependent on the conditions of production.

The here presented biomimetic sensor for food is irreversible and therefore suitable for single use only. Another big advantage of this new sensor system is the cost efficiency for its production. Every step of the sensor production has been carried out on industrial testing equipment and with coating procedures like physical vapour deposition and gravure printing. These production methods guarantee very good economies of scale and thus low cost per unit.

Correlation of colour change and microbiological parameters:

For a functional assay different solutions were prepared as described in Table 1. The respective solutions (1 mm^3) were pipetted onto the sensor surface and then the sensors were incubated in a humidity chamber (over night at 4°C and in parallel also at room temperature). After incubation time the sensor surface was washed with ddH_2O and dried by an airstream.

<Table 1>

Table 1. Pipetting scheme:

	Column 1	**Column 2**	**Column 3**
1	Bacteria-mix* (10^8 CFU/mm^3)	Homogenate II (10^8 CFU/mm^3)	Enzyme-mix** (20 mg/mm^3)
2	Bacteria-mix* (10^6 CFU/mm^3)	Homogenate II (10^6 CFU/mm^3)	Enzyme-mix** (15 mg/mm^3)
3	Homogenate I (10^8 CFU/mm^3)	Pseudomonas (10^7 CFU/mm^3)	Enzyme-mix** (10 mg/mm^3)
4	Homogenate I (10^6 CFU/mm^3)	Pseudomonas (10^6 CFU/mm^3)	Enzyme-mix** (5 mg/mm^3)
5			Ringer Solution (neg. control)

* Equal amount of *Pseudomonas*, *Enterobacter*, *Proteus* and *Salmonella.*

**Equal amount of Proteinase K, Chymotrypsine and Trypsine.

Documentation of the results was done by scanning the sensors on a flat bed scanner (HP Scanjet 4890).

Fig. 4 shows the colour changes as a result of enzymatic degradation of the sensor after overnight incubation at room temperature. This degradation is highly selective and very sensitive; negative control by Ringer solution showed no degradation at all.

Pure bacteria mix and meat homogenate I (incubated for 40 h at 25°C with bacteria mix) with a concentration of 10^6 CFU/mm^3 as was dotted in column 1 did not give signals under these conditions, only meat homogenate I with 10^8 CFU/mm^3 (column 1, line 3) shows a barely visible signal. Homogenate II (incubated for 11 days at 4°C, 10^6 CFU/mm^3) and Pseudomonas (10^7 CFU/mm^3) in column 2 as well as all concentrations of enzyme mix (column 3) show signals clearly visible by the naked eye. The blue dot for the highest enzyme mix concentration results from higher degradation degree than performed by other samples, thus generating another colour.

What looks strange at the first glance in column 2 is the fact that homogenate II (10^8 CFU/mm^3) performed worse than that with the lower count (10^6 CFU/mm^3). The explanation is that seral bacteria levels in the meat juice beyond the logarithmic stage die off and lead to a lower response of the sensor chip than expected. This clearly shows that spot tests as performed here with meat juice containing bacteria in their logarithmic stage are only useful for quick testing of sensors to optimise their properties.

Preliminary experiments show that, for real time monitoring in situ, the sensors have to be kept closely in contact with the meat surface, since only there bacteria will grow freely without substrate depletion. The growing bacteria responsible for meat decay will alter the thickness of the interlayer by excreted lytic enzymes.

As the gained signal is irreversible a colour change can be seen also in case of already died off microorganisms. Studies on this topic are already in progress and will be published soon in details.

<Figure 4>

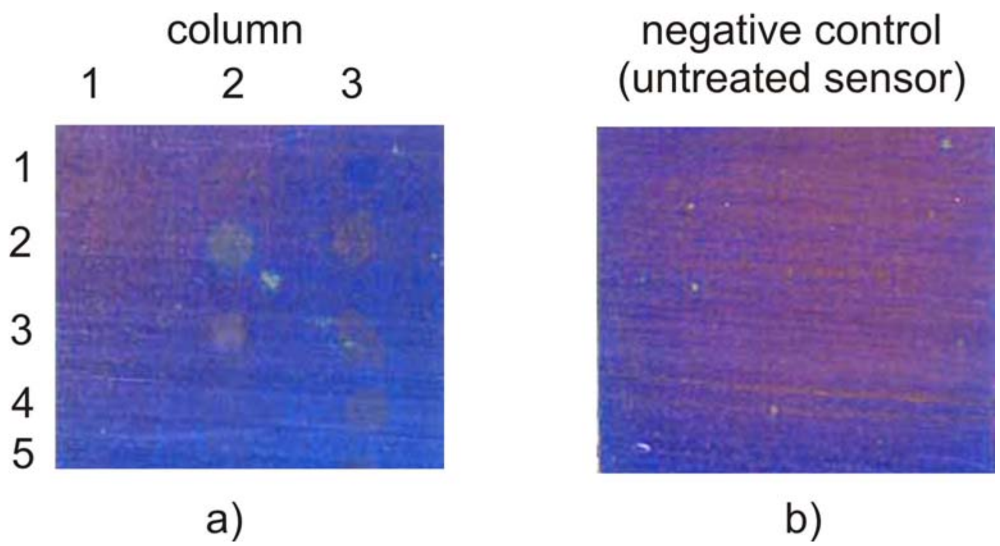


Fig. 4. Sensor response a) after overnight incubation at 25°C b) negative control (untreated sensor). The spots were applied according to the pipetting scheme as given in Table 1. The stripe pattern derives from the gravure printing process.

***Conclusion*:** In this work we could present a biomimetic sensor prototype for monitoring cumulative impacts caused by temperature, bacterial growth and lytic enzyme action. The deterioration of meat can be followed via colour change visible for the naked eye in real time.

This sensor prototype may be adapted for different potential commercial applications as e.g.: facilitated quality control in store and also for shelf life. This gives the consumer insight on the actual freshness of the respective product and the provider the guarantee to having sold intact food. Beyond that the producer can proof optimum hygienic conditions during packaging with the help of the sensor.

Materials and Methods:

The sensor setup is performed according to [8].

Gravure Printing, also known as Intaglio printing, is characteristically used for long run, high quality printing producing a sharp, fine image (Fig. 5). This is accomplished by cutting or engraving and etching various sizes or depths of minute cells (or wells) below the surface of a plate or cylinder. The depth and size of each cell determines the amount of liquid, in our case the biomimetic polymer solution, that can be transferred to the print surface. The cells are flooded and loaded with this solution. The excess is scraped off the surface of the plate by a doctor blade, and what is left in the cells is transferred to the substrate. The nature of the process permits the formation of a layer with the desired thickness. A semi automatic gravure printer for laboratory use from Erichsen (Germany) was used. By this procedure the biomimetic polymer of the distance layer was printed onto the mirror coated substrate.

<Figure 5>

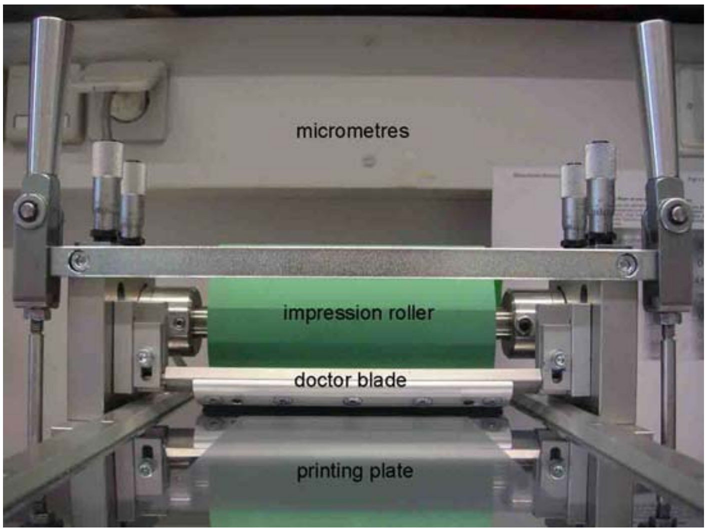


Fig. 5. Gravure Printer (K Printing Proofer 628 by Erichsen)

Sputter Coating: Nanoparticles were applied by an Agar sputter coater as used for electron microscopy. In conventional SEM sputter coating techniques gold is bombarded with heavy gas atoms (Ar). Metal atoms ejected from the Au source by the ionized gas cross the plasma to deposit onto the sensor surface. Thus the nanoparticle layer responsible for the anomalous absorption was generated. This allowed also for visualizing the homogeneity of the printed layers, as changes in thickness of the polymer layer by imperfect printing procedures result in inhomogeneous colours of the sensor.

The whole sensor setup is shown in Fig. 3.

Sampling procedure, strain isolation and growth conditions

Gram-negative aerobic bacterial strains (*Pseudomonas, Enterobacter, Proteus, Salmonella*) were isolated from spoilt meat juice (incubated for 5 h at 37°C) using Salmonella Agar acc. to ÖNÖZ (*Merck*).

<Table 2>

Table 2. Characterisation of microorganism colonies

Microorganisms	**Appearance of colonies**
Pseudomonas	Glossy, dirty yellow to greenish; culture medium is yellow
Enterobacter	Large, mucoid, bluish or reddish, slight precipitation ring around the colonies
Proteus	Rust-coloured, culture medium surrounding the colonies of same colour, if growth is too dense, dark brown to black
Salmonella	Yellow, medium size; 1st day: black dots start to develop on the yellow colonies; 2nd day: black dots clearly visible on the yellow colonies; culture medium surrounding the colonies is yellowish

A colony picked from the selective media was inoculated in 3 cm^3 pepton water (peptone 0.1%, NaCl 0.5%, $Na_2HPO_4.12H_2O$ 0.9% and KH_2PO_4 0.15% all in dd H_2O) and allowed to grow overnight at 37°C. Then 2 cm^3 of the bacterial pre-culture was inoculated in 200 cm^3 pepton water and incubated at 37°C for 24 h. Aliquots (1 cm^3) were frozen in liquid nitrogen and stored at -80°C. After incubation (up to 3 days at 30°C) bacterial counts were determined by plating 100 mm^3 bacterial cultures onto Plate Count Agar (*Fluka*). The culture dilutions (10^{-1} to 10^{-9}) were prepared in Ringer Solution.

Preparation of homogenised infected meat

Pork cutlets (100 g) were infected with nine different bacterial strains that can be found in spoilt meat (*Serratia liquefaciens, Lactobacillus sakei, Lactobacillus curvatus, Leuconostoc mesenteroides, Brochothrix thermosphacta, Acinetobacter lwoffii, Pseudomonas lundensis, Pseudomonas fragi, Pseudomonas fluorescens* from DSMZ, the German Resource Center for Biological Materials), and incubated for 40 h at 25°C (homogenate I) or 11 days at 4°C (homogenate II). After these periods of incubation, the meat was homogenised in 200 cm^3 Ringer Solution using a homogenizer-mixer. Bacterial cocktail was filtered with miracloth and then frozen by liquid nitrogen and stored at -80°C. Bacterial counts were performed, as described above, using the Plate Count Agar (*Fluka*).

Acknowledgements: We gratefully thank AGES (Austrian Agency for Safe Nutrition) for financial support, providing bacterial strains and giving feedback on microbiological problems.

References:

[1] Rodriguez-Mozaz S, Marco M, Lopez de Alda MJ, Barcelo D (2004) Pure Appl Chem 76(49):732

[2] Turner APF, Karube I, Wilson GS (1987) Biosensors: Fundamentals and Applications. Oxford University Press, Oxford, UK

[3] Lowe CR (1985) Biosensors 1 (1):3

[4] Rajinder SS (1994) Biosens Bioelectron 9:243

[5] Eggins BR (2002): Chemical Sensors and Biosensors. Analytical Techniques in the Sciences. 2nd ed, Wiley, Hoboken, US

[6] Kress-Rogers E (1996) Handbook of Biosensors and Electronic Noses: Medicine, Food and the Environment CRC Press, London, UK

[7] Leitner A, Zhao Z, Brunner H, Aussenegg FR, Wokaun A (1993) Appl Opt 32:102

[8] Bauer M, Pittner F (2007) Optical sensor and method for indicating the age or quality of a natural product WO 2007/144 367 A1

Index of Figures

Figure 12. Lipolytic activity screen for Pseudomonas (1) Sallmonella (2) Enterobacter (3) and Proteus (4) using tributyrin basis agar. Incubation at 37 °C for 24 hours.

Figure 13. Lipolytic activity screening for Pseudomonas (1), Sallmonella (2), Enterobacter (3) and Proteus (4), using tributyrin basis agar. Incubation: at 4 °C for three days.

Index of Tables

Table 17a. Experiment with trypton and glucose solved in triflouroethanol as stock solution (50 mg trypton and 10 mg glucose in 10 ml triflouroethanol). PLGA was added later and dissolved at room temperature.

Table 17b. Pipetting scheme. ***** Equal amount of Proteinase K, Chymotrypsine and Trypsine.

Table 18a. Experiment with trypton and glucose solved in ethylacetate (mix for 1-2 hours at 50°C) as stock solution (50 mg trypton and 10 mg glucose in 10 ml triflouroethanol).

Table 18b. Pipetting scheme. ***** Equal amount of proteinase K, chymotrypsin and trypsin.

Table 18c. Pipetting scheme. Ringer Solution as negative control.

Table 19a. Experiment with sunflower oil (1-20 µl/2ml), dissolved in ethylacetate.

Table 19b. Pipetting scheme. ***** Equal amount of proteinase K, chymotrypsine and trypsine.

Table 20a. Experiment with sunflower oil and increased desmodur (triisocyanate) concentration ($5x10^{-3}$ % - 3%), performed in ethylacetate as a solvent.

Table 20b. Pipetting scheme. ***** Equal amount of proteinase K, chymotrypsin and trypsin.

Table 21a. Experiment with vegetable fat (from sunflower, canola and corn) dissolved in ethylacetate at room temperature (55 mg butter/10 ml ethylacetat).

Table 26b. Pipetting scheme. ***** Equal amounts of proteinase K, chymotrypsin and trypsin.

Table 27a. Experiment with tri-natrium-citrate x $2H_2O$ as a substrate, dissolved in ddH_2O (22,8 mg tri-natrium-citrate x $2H_2O$/55 µl ddH_2O).

Table 27b. Pipetting scheme. ***** Equal amount of proteinase K, chymotrypsin and trypsin.

Table 28a. Experiment with lactose (8,6 mg lactose in 10 ml ethyl acetate) and induction of microorganisms to enzyme excretion. Lactose is used as stimulus for lipolytic enzyme secretion especially for the *Lactobacillus* species.

Table 28b. Pipetting scheme. ***** Equal amount of proteinase K, chymotrypsin and trypsin.

Table 29a. Stock of glucose and sunflower oil were prepared in ethyl acetate (7 mg glucose in 10 ml solvent). PLGA sensors whith glucose and oil as a substrate showed the best signal even at 4°C, after only 4 hours.

Table 29b. Pipetting scheme. ***** Equal amount of proteinase K, chymotrypsin and trypsin.

Table 30a. Tween 20 stock solution was prepared in ethyl acetate in 1:100 dilutions.

Table 30b. Pipetting scheme. ***** Equal amount of Proteinase K, Chymotrypsine and Trypsine.

Table 31a. Substrate combination of glucose, lactose and sunflower oil showed excellent results at 4°C after 6 hours.

Index of Diagrams

References

1. Ibrisimovic N, Barth M, Bohrn U, Ibrisimovic M, Pittner F, (2009) *Biomimetic sensor chip monitoring real-time food degradation: correlation of chemical deterioration with microbiological status*.

Chemical Monthly (Special edition), accepted February, 2009 in press.

2. Eggins BR, *Biosensors: an Introduction*. Wiley-Teubner, Leibzig, 1996.
3. Hulanicki , Glab S, Ingman F (1991) Chemical Sensors Definitions and Classification. *Pure and Applied Chemistry* 631247-1250.
4. Lal R (1992) Integrated Biosensors: promises and problems. *Biochemistry and Bioenergetics* 27: 121-139.
5. Rogers KR, Mascini M (1998) Biosensors for field analytical monitoring. *Field analytical chemistry and technology,* 2(6):317-331.
6. Eva Maria Putz (2007) *Diploma Thesis*.
7. Witter LD (1961) Psychrophilic Bacteria-A Review. *Journal of Dairy Science* 44(6):983-1015.
8. Mataragas M, Skandamis PN, Drosinos EH (2008) Risk profiles of pork and poultry meat and risk rating of various pathogen/product combinations. *International Journal of Food Microbiology*, 126:1-12,
9. Ren TJ, Frank JF, Christen GL (1988) Characterization of Lipase of Pseudomonas fluorescens 27 based on Fatty Acid Profiles. *J Dairy Sci*71:1432-1438.

10. Braun P, Balzer G, Fehlhaber K, Activity of bacterial lipases at chilling temperatores. Food Microbiology 18:211-215, 2001.

11. Norris JR, Richmond MH, *Essays in Applied Microbiology, John Wiley & Sons, 1981.*

12. Corry JEL, Curtis GDW, Baird RM, *Handbook pf Culture Media for Food Microbiology,* Progress in industrial microbiology volume 37, 2003.

13. Christen GL, Marshall RT (1984) Selected Properties of Lipase and Protease of Pseudomonas fluorescens 27 Produced in Four Media. *J Dairy Sci* 67:1680-1687.

14. Behrisch R, Andersen HH, Bay HL, Robinson MT, Rosendaal HE,Sigmund P et al. *Sputtering by Particle Bombardement I, Physics and Applications*. Topics in Applied Physics Vol. 47, S. 281, 1979.

15. Knol Ml*, Aufladepotential und Sekundäremission elektronenbestrahlter Körper.* Zeitschrift für technische Physik 16: 467-475, 1935.

16. Rodriguez-Mozaz S, Marco MP, Lopez de Alda MJ Barceló D *Biosensors for environmental applications:* Future development trends. Pure Appl. Chem., 76(4):723–752, 2004.

17. Niemeyer CM, Mirkin CA*, Nanobiotechnology: Concepts, Applications and Perspective*. WILEY-VCH Verlag GmbH & Co. KgaA, Weinheim, 2004*.*

18. Bauer M, Pittner F, Optical sensor *and method for* i*ndicating the age or quality of a na*tural product WO 2007/144 367 A1, 2007.

19. Kumar Ravi MNV, Kumar N, Kumar AJ Domb, Arora M, *Adv. Polym. Sci*., 160, 1-73, 2002.

20. Jain RA, *Biomaterials*, 21, 2475-2490, 2000.

21. Gref R, Minamitake Y, Peracchia MT, et al, *Science*, 263, 1600-1603, 1994.

22. Davda J, Labhasetwar V, *Int. J. Pharm*., 233, 51-59, 2002.

23. Prabha S, Zhou WZ, Panyam J, Labhasetwar V, *Int. J. Pharm*., 244, 105-115, 2002.

24. Sahoo SK, Panyam J, Prabha S, Labhasetwar V, *Controll. Rel*., 82, 105-115, 2002.

25. Panyam WZ, Zhou S, Prabha S, Sahoo SK, Labhasetwar V, *FASEB J.*, 16, 1217-1226, 2002.

26. Leitner A, Zhao Z, Brunner H, Aussenegg FR, Wokaun A Appl Opt 32:102, 1993.

27. Turner APF, Karube I, Wilson GS, *Biosensors: Fundamentals and Applications.* Oxford University Press, Oxford, UK, 1987.

28. Lowe CR, *Biosensors*, 1 (1):3, 1985.

29. Rajinder SS, *Biosens Bioelectron* , 9:243, 1994.

30. Eggins BR: *Chemical Sensors and Biosensors. Analytical Techniques in the Sciences.* 2nd ed, Wiley, Hoboken, US, 2002

Curriculum Vitae

Surame: Ibrisimovic
Firstname: Mirza
Date of birth: January 4th, 1984
Place of birth: Brcko
Natioality: Bosnia and Herzegovina
Gender: Male

Address
Street: Wimmergasse 3/23-24

City, county: Vienna

Postcode: 1050

Country: Austria

Phone no. (home): 0043/069911002037

e-mail address: mirza.ibrisimovic@univie.ac.at ; ibrisimovic2001@yahoo.com

Education

1991 -1992: Elementary School „Nasto Nakic", Brcko, Bosnia and Herzegovina

1992-1993: Elementary School, Maribor, Slovenia

1993-1996: Elementary School „Hamid Berbic" Gornji Rahic, Bosnia and Herzegovina

1996-1999: Elementary School „Arif Dervisevic" Prutace, Bosnia and Herzegovina

1999-2003: Grammar School „Vaso Pelagic“, Brcko, Bosnia and Herzegovina

01.-07.2001: Abroad semester in USA (Ironwood High school in Phoenix, Arizona)

Juni 2003: High School final examination and selection for the best student of the generation.

October 1st, 2003: Start of my Studies in Medicine at Medical University of Vienna

March 1st, 2005: Start of my studies in Molecular Biology at University of Vienna (major subjects: biochemistry, genetics and molecular medicine)

October-November 2007: Advanced Course in Biochemistry “*Optimization of Meat Fresh Sensors on Gold Nanoparticle Basis*“, under supervision of Univ. Prof. Dr. Fritz Pittner (Department for Biochemistry and Molecular Cell Biology; University of Vienna)

March-April 2008: Advanced Course in Developmental Biology: “*Metal Ion Homeostasis in Cells and Organelles; The gating mechanisms in the CorA Mg^{2+}-Channel*”, under supervision of Univ. Prof. Dr. Rudolf Schweyen (Department of Genetics; University of Vienna)

June-August 2008: Elective Laboratory Course (Molecular Medicine)-Part 1and Part 2: “*Molecular mechanisms involved in the transport of VLDL from the egg yolk to the embryo proper; Localisation and cloning of LRP-380; R/O Genotyping*”, under supervision of Univ. Prof. Dr. Marcela Hermann (Department of Medical Biochemistry, Division Molecular Genetics; University of Vienna)

October, 2008: Start of my Diploma thesis: *„Evaluation of novel, nanotechnological biosensor-chips for monitoring of bacterial spoilage: Evaluation of sensor sensitivity and correlation with currently used microbiological testing methods and their improvement”.*

WS 2008/09: Tutor in Laboratory Course C + Advanced Laboratory Course in Biochemistry – Protein biochemistry; Department for Biochemistry and Molecular Cell Biology; University of Vienna

SS 2009: Tutor in Laboratory Course C + Advanced Laboratory Course in Biochemistry – Protein biochemistry; Department for Biochemistry and Molecular Cell Biology; University of Vienna.

January 30th, 2009: Successful completion of the 1st part of the 2nd Diploma exam

Publication:

Ibrisimovic N, Barth M, Bohrn U, Ibrisimovic M, Pittner F, (2009) *Biomimetic sensor chip monitoring real-time food degradation: correlation of chemical deterioration with microbiological status*.

Chemical Monthly (Special edition), accepted February, 2009 in press

October, 2008-February, 2009: “Test Tool-Food Microbiology” invention.

2009: “Two-Layer (PLGA) Biosensor” patentable invention (together with Univ.- Prof. Dr. Fritz Pittner, Mag. Nadira Ibrisimovic, Margith Barth and Ulrich Bohrn).

Other qualifications

Language: Bosnian; Croatian; Serbian (native languages)

Englisch: fluent spoken and written

German: fluent spoken and written

Excellent computer skills.